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VOL. 49 FEBRUARY, 1957 No. 2

CLINICAL AND EXPERIMENTAL

THE VOLUME OF DISTRIBUTION OF HIGH MOLECULAR WEIGHT DEXTRAN* AND ITS RELATION TO PLASMA VOLUME IN MAN

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THE dilution of Evans Blue dye (T-1824) and I^{131}-labeled human serum albumin has gained wide acceptance as a measure of plasma volume. In studies using each label simultaneously, the volumes of distribution are equal.[1, 2] It has been demonstrated that the "first circulation" distribution of each is similar.[3] However, both methods depend upon the tagging of serum albumin, and, in essence, it is the early volume distribution of the labeled albumin which is estimated. In diseases associated with increased vascular permeability, methods dependent upon albumin distribution may overestimate plasma volume.

For plasma volume studies, it would be desirable to use a substance which does not form a complex with circulating proteins. The fractionation of dextran yields a polysaccharide which has a molecular weight much greater than most plasma proteins. Even in pathologic states, such macromolecules probably would be retained better than albumin, and would circulate independently. Although dextran is known for its property as a plasma expander, injection of small amounts would not produce significant increase in plasma volume.

This paper describes the method for estimating the volume distribution of high molecular weight dextran. The space measured is reproducible and has

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**Public Health Service Research Fellow of the National Cancer Institute.

other characteristics which would seem to indicate that plasma volume is being measured. Consideration is given to the possible advantages of dextran dilution over tagged albumin methods in determining plasma volume.

METHODS

Measurement of the serum dextran concentration was carried out by a modification of the method of Bloom and Wilcox.[4, 5] Since serum dextran concentrations were expected to be below 100 mg. per cent, it was necessary that the serum blank be negligible. Potassium hydroxide digestion of the serum was carried out as described. The alcohol precipitation of the dextran was altered slightly. Table I shows the blank values and recoveries obtained using alcohol concentrations from 44 to 79 per cent. Between 63 and 70 per cent of the blank values become high and recoveries unsatisfactory. Because of the variable amount of total solution remaining after digestion, an alcohol concentration of 57 per cent (10 ml. of water and 18 ml. of 95 per cent ethanol added to the digested solution) was chosen as a suitable medium for the dextran precipitation.

TABLE I. EFFECT OF ALCOHOL CONCENTRATION ON THE PRECIPITATION OF DEXTRAN

ALCOHOL CONCENTRATE (PER CENT)	SERUM BLANK (MG. PER CENT)	SERUM + DEXTRAN* (MG. PER CENT)
44	2.7	74.6
57	2.2	77.0
63	3.4	77.5
70	32.9	97.0
79	20.3	91.5

*Theoretical concentration 75 mg. per cent.

After refrigeration and centrifugation the redissolved precipitated material was made to a 50 ml. volume. This solution was shaken vigorously and poured into clean tubes for recentrifugation. It was found that if recentrifugation were omitted, the blank values would be more variable and slightly higher. There was some material which was not water soluble but which contributed to the subsequent color reaction. After recentrifugation this material could be seen in the bottom of the tubes. These two modifications produced blank values in fresh serum of 2.2 ± 1.2 mg. per cent. Bloom and Wilcox[5] reported blanks averaging 12.4 ± 3.2 mg. per cent.

Color was developed by the addition of the anthrone reagent. This reagent was prepared fresh each day by dissolving anthrone in cold 95 per cent sulfuric acid. All unknowns were done in duplicate, and a glucose standard and serum blanks were run in triplicate with each set of unknowns. Water blanks treated in the same manner were used to set the Evelyn colorimeter at 100 per cent transmittance with a 620 mμ filter. The anthrone reagent was delivered cold from a 10 ml. glass-tipped syringe mounted on a bracket. In this way the anthrone reagent could be added forcefully and accurately into an ignition tube containing the unknown. The tube was immediately immersed into a boiling water bath for six minutes, cooled, and the color read. All tubes were individually timed.

Under these conditions color formation was found to be maximum after five to eight minutes of heating. The peak color formation when the anthrone was at room temperature occurred earlier and disappeared sooner than it did with cold reagent. Use of the cold reagent permitted more latitude for reagent variation and timing errors than did use of warm reagent.

Recovery of dextran added to serum in concentrations of 60 to 90 mg. per cent averaged 99.9 per cent (Table II). Estimation of the error for the methods from the recovery experiment gave ± 1.9 per cent as the coefficient of variation. Repeated determinations of the dextran solution used for injection showed a coefficient of variation of ± 2.0 per cent at an average concentration of 6.29 Gm. per cent.

Dextran for injection was made by dissolving weighed, dry dextran in 0.85 Gm. per cent saline to make a 6 Gm. per cent solution. This dextran solution was filtered and sterilized immediately. After a blood specimen to be used as the serum blank was obtained, 35 to 40 ml. of the dextran solution was injected from a 50 ml. syringe through a three-way stopcock.

TABLE II. RECOVERY OF DEXTRAN ADDED TO SERUM

THEORETICAL CONCENTRATE (MG. PER CENT)	FOUND (MG. PER CENT)	RECOVERY (PER CENT)	RANGE (MG. PER CENT)	NUMBER OF DETERMINATIONS
60	60.8	101.3	59.5- 61.0	4
70	69.1	98.7	67.5- 71.0	4
80	79.7	99.6	78.5- 82.0	4
100	99.9	99.9	99.5-100.5	4

Average 99.9 ± 1.4 per cent.

Injection was timed so that the entire volume was delivered in one minute. The syringe was weighed before and after injection to 0.1 Gm. All blood specimens were drawn from veins other than the one used for the injection. The blood was immediately centrifuged and the serum removed. If the dextran determination were not to be begun the same day, the serum would be sealed in ampules and frozen until the determination was done. With each set of sera, a sample of the injected dextran was run to determine the concentration of dextran given.

All subjects were healthy young male students or physicians. The subjects for the first part of the study were not at rest before the test. Although they were recumbent during the injection, they were sitting or active during the subsequent period of blood collection. The eleven subjects used in the latter part rested in a horizontal position for at least one hour before the beginning of the test and also during the period of blood collection.

RESULTS

Two experiments were carried out on one subject using clinical dextran for injection (average molecular weight 70,000; low fraction, representing 8.2 per cent of total, 27,600; high 9.6 per cent, 180,000). It was obvious that this

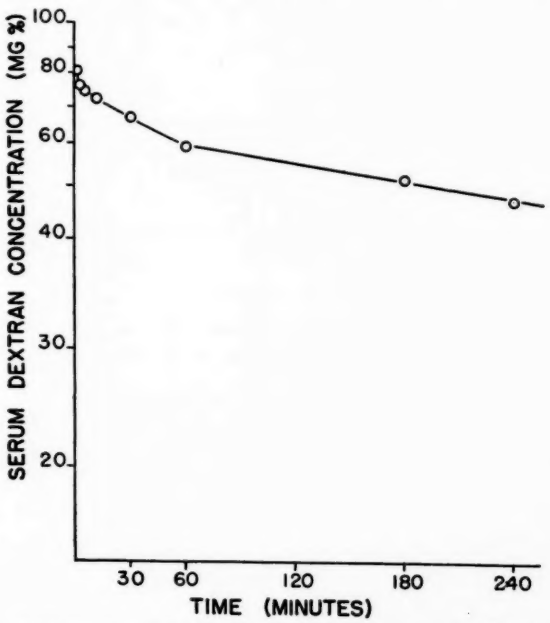

Fig. 1.—Serum dextran concentration following intravenous injection of clinical dextran.

material was unsuitable for volume distribution measurements because of the nonlinearity of the serum levels during the early part of the curve (Fig. 1). Urinary excretion of the dextran amounted to 11 per cent of the injected dose during the first hour and an additional 7 per cent during the second hour.

The remainder of the study was carried out using high molecular weight dextran (N.R.C. fraction No. 6, average molecular weight 194,900; 4.8 per cent, 129,000; 7.4 per cent, 232,500). Two experiments on each of three individuals demonstrated that from five minutes to four hours after injection, serum levels of dextran fell at a slow exponential rate of 2.2 per cent per hour. One study in another subject indicated that this disappearance rate may be maintained for as long as 48 hours. The levels of dextran were variable in the one- and three-minute samples (Fig. 2). Urine excretion of dextran over the four-hour period averaged 1.5 per cent of the injected dose (range 1.0 to 2.3 per cent).

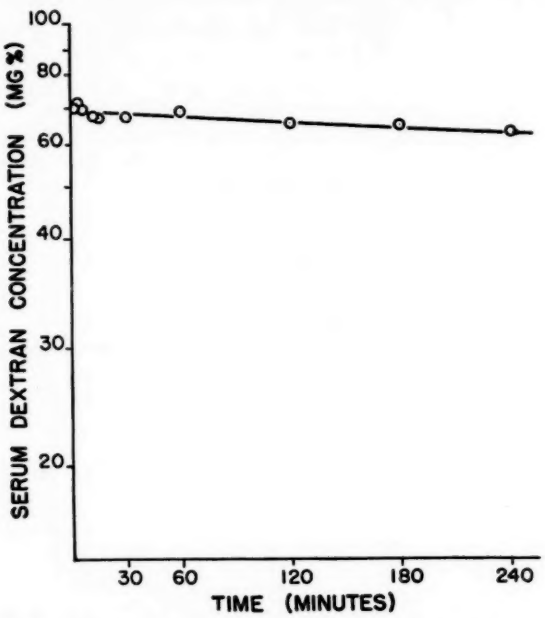

Fig. 2.—Serum dextran concentration following intravenous injection of high molecular weight dextran.

To test the reproducibility of the method, we measured the volume of distribution in six individuals on more than one occasion. Except for the three subjects mentioned above, blood samples were taken 5, 10, 15, 30, and 60 minutes after the end of injection. Extrapolation of the straight line obtained from the semilogarithmic plot of the serum values was made to estimate the concentration at theoretical time zero. An evaluation of the results presented in

TABLE III. REPRODUCIBILITY OF APPARENT VOLUME OF DISTRIBUTION OF DEXTRAN

SUBJECT	WT. (KG.)	HCT. (PER CENT)	VOLUME OF DISTRIBUTION REPEATED DETERMINATIONS (ML.)				AVERAGE (ML.)
A. B. C.	69.2	43.4	3,230	2,760	2,880	2,970	2,870
J. C.	75.0	45.8	2,790	2,700			2,745
J. J.	78.0	43.3	2,900	2,860			2,880
A. R. T.	66.0	44.0	2,400	2,330	2,410		2,380
W. C.	80.5	47.6	2,710	2,680			2,695
R. T.	74.0	44.0	3,760	3,660			3,710
						Average	2,880 ml.

Standard deviation of volume of distribution from individual mean volume = ±96 ml. (±1.3 ml./kg.). Coefficient variation ±3.3 per cent.

Table III indicates that any one measurement of dextran space has a reliability as described by a standard deviation of ±96 ml. (±1.3 ml./kg.) from each individual's mean volume. This amounts to ±3.3 per cent of the volume measured.

Additional examination of the method was carried out by observing the arterio-venous difference during the early phase of distribution of dextran. As a part of a different project hemodynamic studies were first carried out on a patient by cardiac catheterization. The patient had undergone a mitral valvotomy three months previously but was free of signs of failure at the time of this examination. Dextran was injected rapidly (20 seconds) through the catheter which was placed in the right ventricle. Arterial and venous samples were obtained from the arm opposite the site of introduction of the catheter. No significant arterio-venous difference could be noted after three minutes from the end of injection. It was not possible to estimate accurately the exact time of complete mixing because there were no samples earlier than one minute.

To study the excretion of this dextran fraction in a disease associated with increased glomerular capillary permeability, we gave dextran to a patient who was in the nephrotic stage of chronic glomerulonephritis. Albuminuria averaged 2.4 Gm. per day. Two hours after the administration of dextran he had excreted only 0.5 per cent of the injected dose. Urine volume during these two hours was 393 ml.

Finally, the dextran space of 11 normal men was determined. The average volume of distribution was 3,150 ml., or 41.0 per kilogram of body weight in these resting subjects (Table IV). The average absolute dextran space showed a coefficient of variation of ±13 per cent. Correction for body weight did not decrease the coefficient of variation. However, the sample was too homogeneous to test the effectiveness of correction for weight, height, or surface area.

TABLE IV. APPARENT VOLUME OF DISTRIBUTION OF DEXTRAN IN RESTING NORMAL ADULT MALES

SUBJECT	WT. (KG.)	HT. (CM.)	HCT. (%)	DEXTRAN SPACE	
				ML.	ML./KG.
A. R. T.	66.0	178	44.0	2,410	36.5
D. N.	90.3	176	41.4	3,850	42.6
R. T.	74.0	176	44.0	3,710	50.2
R. C.	88.2	185		3,410	38.7
H. V.	77.5	182	41.8	3,140	40.5
F. F.	88.4	179	46.4	3,030	34.2
J. C.	79.9	179	46.3	3,220	40.3
M. M.	79.0	178	45.5	3,240	40.9
J. I.	83.5	180	45.7	3.140	37.6
S. H.	56.5	172	46.8	2,750	48.7
J. L.	67.4	176	41.1	2,770	41.1
Mean	77.3	178	44.3	3,150	41.0
Median	77.5	178	45.5	3,140	40.3
Standard deviation	±8.9	±3	±2.3	±420	±4.8
Coefficient variation	±12%	±2%	±5%	±13%	±12%

DISCUSSION

The preliminary work with the lower molecular weight "clinical dextran" demonstrates the unsuitability of this material for plasma volume measurement. Similar material was used by Birch[6] and his results indicate the difficulties. High molecular weight fractions are retained in the vascular bed

much more effectively than the lower weight fractions.[7-10] Although there seems to be some vascular escape of the higher weight fractions, all of the animal work has been done by injecting large volumes of dextran (10 to 40 ml. per kilogram). Wasserman and Mayerson[9] pointed out that large infusions result in less intravascular retention than do smaller infusions of the same molecular weight. This probably is explained by the expansion of the vascular network and mechanical leakage. Our use of a maximum amount of 0.6 ml. per kilogram would not be expected to produce significant expansion.

The use of small amounts of dextran demands that concentrations below 100 mg. per cent must be measured accurately and that serum blanks be negligible. The reduction of the serum blanks was due mostly to alteration of the alcohol concentration used to precipitate the dextran from solution. In contrast to the findings of other investigators we have found that recoveries are satisfactory at this alcohol concentration in an alkaline medium.[11, 12]

It has been demonstrated that the heating time of the anthrone sulfuric acid reagent with the specimen is critical.[13] For this reason we have preferred to start the reaction immediately upon the addition of the anthrone reagent rather than to layer the reagent and then mix. Optimal heating time should be determined for the conditions of the procedure in each laboratory.

Although there is no direct method of proving that a substance measures true plasma volume in humans, dextran fulfills certain theoretical criteria.[14] Distribution of dextran was rapid and the time for distribution compares with other materials mixed in the same volume.[15, 16] Extravascular loss was probably small over a long period. During the four hours after injection 1.5 per cent of the dextran was recovered in the urine. Even with albuminuria, dextran was not found in the urine in increased amounts. In normal subjects 0.7 per cent of the injected dose was found in the urine at the end of the first hour as compared with 1.5 per cent in four hours. This indicates that there was slight differential extravascular escape, which could not be detected by any change of the slope of the disappearance curve. Although the disappearance curve was not examined carefully beyond four hours, one experience indicates that dextran may disappear at a constant rate for as long as 48 hours. After equilibration, no arterio-venous difference of dextran concentration could be demonstrated.

The disappearance of dextran after equilibration can be accounted for partly by renal excretion. Slow metabolism of dextran is known to occur in animals and humans,[17-19] and this undoubtedly accounts for another part of the disappearance rate. A third possibility is the extraction of dextran by the tissues. Even with lower molecular weight dextran this occurs some time after injection[20-22] and probably is not a factor in the measurement of the volume of distribution.

The average volume of distribution in eleven resting normal subjects was 41.0 ml. per kilogram of body weight. This is about the same value found for I^{131}-human serum albumin distribution and for more recent studies with T-1824.[2, 23-25] The coefficient of variation from the average figure was of the same magnitude as reported with other methods. A more critical comparison of the dextran and tagged albumin methods is reported in the following paper.[26]

SUMMARY

The volume of distribution of high molecular weight dextran has been examined in normal men. The characteristics and volume of distribution make it likely that this dextran is diluted in the plasma volume.

We wish to acknowledge the assistance of Dr. Paul Yu, Dr. Howard Joos, and Dr. Frank Lovejoy in examining arterio-venous difference of injected dextran.

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THE RELATIVE VOLUMES OF DISTRIBUTION OF I^{131}-TAGGED ALBUMIN AND HIGH MOLECULAR WEIGHT DEXTRAN IN NORMAL SUBJECTS AND PATIENTS WITH HEART DISEASE

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THE development and refinement of albumin-tagging methods in recent years has stimulated study of plasma volume alterations in both normal and diseased persons. The validity of such studies is dependent upon the assumption that the volume of distribution of tagged albumin, following intravascular mixing, is equal to the plasma volume, and that this relationship is not altered in any of the variety of pathologic states which have been studied. Although this assumption has been repeatedly questioned, comparative studies of the volumes of distribution of albumin and molecules of significantly larger size have been contradictory, and they have been performed only in dogs[1-3] and normal human subjects.[4]

The refinement of the analytic methods for dextran and the demonstration of its applicability to plasma volume measurement[5] have led to such a comparative study. In view of the interest and significance attached to plasma volume changes in congestive heart failure, simultaneous determinations of high molecular weight dextran and I^{131}-tagged albumin spaces were performed in a group of patients with heart failure and were repeated in certain of the same subjects following cardiac compensation. For comparative purposes an identical study has been conducted on a group of normal human subjects.

MATERIALS AND METHODS

The 10 normal subjects studied were healthy young physicians who showed no evidence of cardiac, hepatic, or renal disease. Fifteen patients were selected who showed unequivocal evidence of severe right-sided congestive heart failure manifested by increased venous pressure, hepatomegaly, and marked peripheral edema. Pulmonary congestion was also present in the majority of patients studied. All had rheumatic, arteriosclerotic, or hypertensive heart disease, or a combination of these. Eight of these patients were restudied at a later time when optimal cardiac compensation had been achieved by standard therapy. Although free of gross edema at the time of restudy, all were still significantly incapacitated by their cardiac disease.

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All subjects remained recumbent or semirecumbent in bed for a minimum of one hour prior to the test and throughout the period of study. Immediately sequential rapid injections of radioiodinated serum albumin (RISA)* and dextran were given through a three-way stopcock and a No. 18 needle, which had been placed previously in an arm vein, and subsequently flushed with 5 per cent dextrose in water solution. During the first phase of this study, venous blood samples were drawn in the patients with heart disease at 20, 30, 40, and 50 minutes after injection, and at 10, 20, 30, and 40 minutes in normal subjects. Plasma volume was calculated by extrapolation to "zero" time. Nine patients with edema, 5 with treated heart disease, and 3 normal subjects were studied in this manner. In the remaining determinations a single blood specimen was drawn at 20 minutes in the cardiac patients and at 10 minutes in normal subjects, and plasma volume was calculated directly from this specimen. Since we were mainly concerned with the relative volumes of distribution of the two test materials, no correction was attempted for the slope of the disappearance curves during the period of mixing.

High molecular weight dextran (N. R. C. fraction No. 6, average molecular weight 194,900) was used throughout the study. It was prepared, injected, and analyzed as outlined in the preceding paper.[5] When plasma volume was calculated from a single blood specimen, precipitation of dextran was performed in triplicate, and duplicate color reactions carried out on each. Serum blanks were always determined in triplicate.

RISA was diluted in normal saline to a concentration of approximately 2 μc per milliliter prior to injection; a total dose of approximately 5 μc was administered. Injection was made with a 5 ml. syringe which was weighed to 0.001 Gm. prior to and after injection. Counting standards were prepared by delivery of an aliquot of the same RISA solution with a 5 ml. syringe into a 1 L. volumetric flask. It was made up to volume with a dilute plasma in water solution, mixed rapidly, and immediately pipetted into counting vials. The syringe was weighed, using the same procedure as with the test dose syringe. The problem of adsorption of the RISA onto the walls of the syringes was investigated. No discrepancy could be demonstrated in the loss of activity between the syringe used for the test dose and the syringe used for making the counting standard. The use of a dilute protein solution for preparing the standard, and the subsequent rapid mixing and pipetting were employed to prevent loss of activity onto the walls of the volumetric flask. Duplicate counts on the standard solutions uniformly showed less than 1 per cent variation. A plasma blank was taken prior to injection in all subjects restudied with RISA, and subsequent samples corrected for residual activity. Blood samples were heparinized and the plasma was separated by centrifugation and immediately transferred into counting vials with scrupulously clean, calibrated pipettes.

Counting was performed in a well-type scintillation counter in calibrated vials, with appropriate corrections made for background activity. Sufficient counts were recorded on each specimen to assure a counting error of less than 1 per cent.

RESULTS

A. *Disappearance Curves.*—Calculations based on the extrapolated value at "zero" time revealed a mean disappearance in subjects with heart disease of 2.8 per cent of administered dextran and 3.1 per cent of the tagged albumin in twenty minutes. This discrepancy is not statistically significant. The disappearance rates of the two substances did not differ significantly in either the patients with heart failure, the treated cardiac patients, or the three normal subjects.

Apparent complete intravascular mixing of dextran and RISA had occurred in twenty minutes in all cardiac patients so studied. Blood concentrations subsequently fell at a constant exponential rate to the time of the last blood sample at fifty minutes.

*RISA was obtained from Abbott Laboratories.

TABLE I. SIMULTANEOUS DEXTRAN AND TAGGED ALBUMIN SPACES IN TEN NORMAL SUBJECTS

SUBJECT	BODY WEIGHT (KG.)	PLASMA VOLUME (LITERS)		DIFFERENCE RISA-DEXTRAN (LITERS)	PER CENT* DIFFERENCE	PLASMA VOLUME (ML./KG. BODY WT.)	
		DEXTRAN	RISA			DEXTRAN	RISA
B. S.	100.2	2.79	3.12	0.33	11.8	27.8	31.1
R. S.	87.0	4.19	4.63	0.44	10.5	48.2	53.2
R. M.	77.2	3.27	3.50	0.23	7.0	42.4	45.3
M. L.	70.1	2.63	2.78	0.15	5.7	37.5	39.7
E. H.	70.3	2.61	2.76	0.15	5.7	37.1	39.3
G. G.	87.3	3.52	3.70	0.18	5.1	40.3	42.4
S. D.	70.5	2.93	3.07	0.14	4.8	41.6	43.5
L. C.	69.0	2.62	2.73	0.11	4.2	38.0	39.6
R. B.	71.2	2.85	2.96	0.11	3.9	40.0	41.6
J. G.	72.0	3.35	3.39	0.04	1.2	46.5	47.1
				Mean	6.0	39.9	42.3
				Standard deviation	±3.1	±5.6	±5.8
				Standard error	±1.0	±1.75	±1.8

*Calculated as difference between albumin and dextran plasma volumes (Δ) divided by dextran plasma volume multiplied by one hundred:

$$\% \text{ Difference} = \frac{\Delta}{\text{Dextran P.V.}} \times 100$$

B. *Normal Subjects.*—Table I summarizes the results of simultaneous dextran and RISA plasma volume determinations in ten normal subjects. In every instance plasma volume measured by RISA dilution was larger than that determined by dextran. The mean difference between the two methods for the group, 6.0 per cent, was highly significant statistically ($P = <0.01$).

Expressed in relation to body weight, the mean dextran plasma volume does not differ significantly from a group of eleven normal subjects similarly studied by Craig and Waterhouse.[5] Although the group is relatively small, the mean tagged albumin space in milliliter per kilogram of body weight agrees closely with reports from other laboratories.[6, 7]

C. *Congestive Heart Failure With Edema.*—Sixteen studies were performed on 15 patients with congestive heart failure. The results are presented in Table II. With the exception of J. H. the tagged albumin space exceeded the dextran space in every subject, with a mean discrepancy of 10.2 per cent. The magnitude of the discrepancy between the two methods could not be correlated with the size of the plasma volume or the severity or type of heart disease present.

Calculations of plasma volume related to edema-free body weight are presented. Only in Subject A. C. does this figure appear to fall clearly in the normal range. He was inadvertently studied at a time when he was undergoing a mercurial diuresis, during which a considerable decrease in plasma volume may occur.[8] Despite the discrepancy between the RISA and dextran plasma volumes, it is of note that plasma volume was markedly expanded in most patients and mean plasma volumes for the group were significantly above normal with either method of determination.

D. *Treated Heart Failure.*—Plasma volume studies were repeated on eight subjects following diuresis and loss of peripheral edema (Table II). In

TABLE II. DEXTRAN AND TAGGED ALBUMIN PLASMA VOLUME DETERMINATIONS IN PATIENTS WITH CARDIAC FAILURE AND EDEMA, BEFORE AND AFTER THERAPY

			HEART FAILURE WITH EDEMA								AFTER THERAPY—EDEMA FREE					
				BODY WEIGHT (KILOGRAMS)		PLASMA VOLUME (LITERS)		DIFFERENCE (PER CENT†)	PLASMA VOLUME (ML./KG.)‡			PLASMA VOLUME (LITERS)		DEFFERENCE (PER CENT†)	PLASMA VOLUME (ML./KG.)‡	
SUBJECT	AGE	SEX	DATE	FAILURE	EDEMA FREE	DEXTRAN	RISA		DEXTRAN	RISA	DATE	DEXTRAN	RISA		DEXTRAN	RISA
J. G.	66	M	11/22/55	56.6	52.1	2.79	3.08	10.4	53.6	59.1	12/ 6/55	2.06	2.35	14.1	39.5	45.1
F. G.	82	M	12/10/55	114.0	91.0	5.42	5.92	9.2	59.6	65.1	12/23/55	3.68	4.05	10.0	40.4	44.5
					91.0						10/21/55	3.65	4.00	9.6	40.1	44.0
C. H.	68	M	10/25/55	76.0	69.2	3.74	4.24	13.4	54.0	61.3	11/ 4/55	3.26	3.49	7.1	47.1	50.4
J. N.	80	M	10/29/55	70.2	62.2	4.59	5.09	10.9	73.8	81.8	11/ 8/55	3.90	4.33	11.0	62.7	69.6
W. P.	64	M	12/30/55	78.0	63.4	4.64	5.02	8.2	73.2	79.2	1/13/56	4.57	4.82	5.5	72.1	76.0
J. H.	84	M	1/17/56	73.4	63.2	4.47	4.47	0	70.7	70.7	1/27/56	3.76	4.11	9.3	59.5	65.0
M. S.	40	M	10/13/55	67.0	63.0	3.11	3.65	17.4	49.4	57.9						
			1/ 6/56	65.0	56.0	4.52	4.79	6.0	80.7	85.5	1/25/56	4.03	4.27	6.0	72.0	76.2
A. C.	73	M	2/ 7/56	90.0	81.2	3.35	3.59	7.2	41.3	44.2	2/15/56	3.16	3.49	10.4	38.9	43.0
M. H.	65	F	10/11/55	76.6	*	3.52	3.95	12.2	*	*		Mean		9.2	52.5	57.1
F. S.	82	M	11/26/55	79.0	*	3.32	3.63	9.3	*	*		Standard deviation		±2.7	±14.1	±14.4
S. H.	67	F	12/29/55	68.2	55.5	3.50	3.81	8.9	63.1	68.6						
M. R.	53	M	1/ 3/56	77.0	*	3.57	4.06	13.7	*	*		Standard error		±0.9	±4.7	±4.8
J. D.	69	F	3/ 9/56	74.5	63.2	3.09	3.41	10.4	48.9	54.0						
I. P.	80	M	4/18/56	*	*	3.15	3.58	13.7	*	*						
J. A.	78	M	4/24/56	74.0	58.0	3.44	3.89	13.1	59.3	67.1						
						Mean		10.2	60.6	66.2						
						Standard deviation		±4.0	±12.0	±11.7						
						Standard error		±1.0	±3.5	±3.4						

*Patient died in heart failure. Dry weight not obtainable.
†Percentage difference between dextran and albumin plasma volumes calculated as in Table I.
‡Calculated on the basis of edema-free body weight.

all but two, W. P. and A. C., a significant fall in plasma volume occurred. Concomitant Laennec's cirrhosis in Patient W. P. may have accounted for the maintenance of a high plasma volume following cardiac compensation. As mentioned previously, it is probable that the plasma volumes obtained on A. C. during heart failure were spuriously low.

As in the other groups studied, a discrepancy between the RISA and dextran plasma volumes was found, with a mean difference of 9.2 per cent. In individual subjects no consistent change in this discrepancy occurred following treatment of the heart failure.

Considerable variation in plasma volume is apparent in the compensated cardiac patients. While some manifested a return to an apparently normal volume, others showed a persistent significant elevation at the time of restudy. The relatively small number of subjects studied and the clinical heterogeneity of the patients preclude any definitive conclusions on this point.

E. *Comparison Between Groups.*—As has been noted, in all groups studied the tagged albumin space was significantly larger than the dextran space. In the normal subjects this discrepancy was smaller than in the cardiac failure

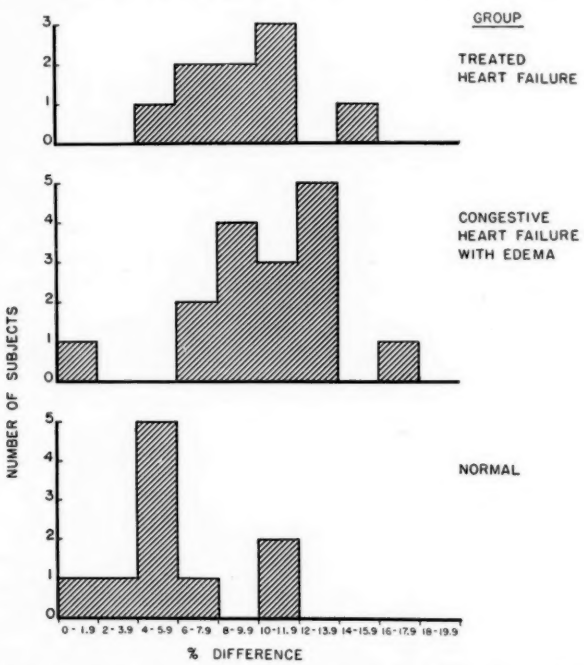

Fig. 1.—Frequency distribution of individual differences in dextran and albumin plasma volumes, arranged by groups of subjects studied

group ($P = <0.01$ and in the treated cardiac patients ($P = <0.04$). In contrast, the discrepancy between the two methods did not differ significantly on comparison of the group with congestive heart failure with patients with treated heart disease.

Fig. 1 depicts graphically the differences between tagged albumin and dextran plasma volumes in individual subjects within the three groups studied.

Since the disappearance rates of dextran and RISA did not differ in the present study, the later determinations were performed with a single blood sample. The discrepancy between the dextran and albumin spaces persisted, and the magnitude of this discrepancy was unchanged from determinations done by means of multiple blood samples and extrapolation of the disappearance curves.

DISCUSSION

Although the dilution of tagged albumin has become widely accepted as a valid measure of plasma volume, ample evidence exists to indicate that albumin and other macromolecular substances diffuse readily from the vascular compartment into the lymphatics. Radioactive albumin appears in the thoracic duct lymph of dogs[9, 10] and man[11] by ten minutes after its intravenous injection. That the amount detected in the thoracic duct at this time is only a minute fraction of the injected dose has been cited as evidence against the occurrence of any significant loss of intravascular tagged albumin to the lymph during this period.[11] However, since the time lag between the escape of tagged albumin to the lymph and its appearance in the thoracic duct at the neck, as well as the volume of the lymphatic system involved, is unknown, it is not possible to quantitate the early loss of the test material by this means. In addition, the possibility that there is a rapid equilibration of tagged albumin between the plasma and any portion of the extravascular fluid space cannot be excluded by this means. In view of the inability to perform direct measurements, any conclusions as to whether or not this loss is of sufficient magnitude to affect significantly the measurement of plasma volume with tagged albumin are speculative.

The studies of Wasserman and his co-workers[12] indicate that the plasma to lymph concentration gradients of various dextran fractions are directly related to the molecular weight of the fraction studied. This relationship between molecular weight and diffusion into the lymph appears to apply also to the plasma proteins, albumin and globulin.[13, 14] These observations suggest that the magnitude of the early loss of injected tagged albumin might be assessed indirectly by relating this loss to that of a larger molecule, which, from experimental evidence, can be expected to diffuse from the circulation at a slower rate.

The results of the present study suggest that during the test period, tagged albumin leaves the circulation in significantly larger amounts than high molecular weight dextran. This results in a falsely high plasma volume estimation based on albumin dilution. It appears likely that this differential escape occurs during the mixing periods of the test materials. If the difference in the volumes of distribution of dextran and albumin resulted from a constant increased rate of loss of albumin throughout the period of blood sampling, extrapolation to "zero" time will eliminate this difference. In contrast, in the present study a discrepancy between the albumin and dextran spaces was found in all subjects studied by means of extrapolation of the disappearance

curves, and the magnitude of this discrepancy was unchanged in determinations made from a single blood sample. In addition, the lack of correlation between the slopes of the disappearance curves and the relative volumes of distribution of albumin and dextran is further evidence that the calculated increased escape of tagged albumin from the vascular space occurred soon after injection, before mixing was complete. It is postulated that tagged albumin equilibrates rapidly with an extravascular fluid pool and, therefore, that this loss of activity is not reflected in the disappearance slope. Dextran escapes less readily into this hypothetical space and consequently the volume of distribution of dextran is smaller than that of tagged albumin.

The measures utilized to eliminate the known sources of error in the present experiment have already been enumerated. The data showing that the mean discrepancies between the RISA and dextran plasma volumes differed in the normal and cardiac groups would confirm the conclusion that a constant technical error was not responsible for this observed difference in the volumes of distribution of the two test materials.

Certain factors present in congestive heart failure have been shown to influence the rate of diffusion of large molecules from the plasma to the lymph. Expansion of plasma volume accelerates lymphatic flow and decreases the plasma to lymph concentration gradient of dextran and albumin.[12] Similarly, a rise in venous pressure in the limb of the dog results in an increased flow of lymph and total lymphatic protein content.[15] Compatible with these findings is the observation that the rate of lymph flow is markedly increased in heart failure.[16] It is attractive to relate the increased loss of tagged albumin from the circulation of patients in this study with cardiac failure to the elevated plasma volume and venous pressure present in these subjects. However, since the correction of venous congestion and edema, and the restoration, in some patients, of a normal plasma volume failed to affect the observed discrepancy between the albumin and dextran spaces, it is possible that other more subtle factors, such as tissue hypoxia, may be important in this regard. It should be emphasized that these patients were not normal at the time of restudy; they remained moderately to severely handicapped by their cardiac disease. It must be concluded that the finding of an increment in the loss of albumin from the circulation in patients with heart failure as related to normal subjects, and the failure to correct this deviation from the normal following apparent compensation remain essentially unexplained.

Overestimation of the plasma volume is one of the factors which will materially affect the calculation of the total body to venous hematocrit ratio. Recent studies utilizing tagged erythrocytes and albumin for the measurement of red cell mass and plasma volume reveal an average total body venous/hematocrit ratio of 0.91 to 0.93 in normal subjects.[6, 17, 18] Our data suggest that this ratio may be somewhat higher, but they do not invalidate the concept that the capillary hematocrit is lower than the hematocrit in the large vessels. For example, calculations based on a 6 per cent overestimation of plasma volume by

albumin dilution will result in a revision of the normal ratio from 0.92 to approximately 0.95. It is not proposed that the latter is necessarily a valid figure, however, since other factors affecting the calculation of this ratio have not been investigated in the present study. Schreiber and associates[6] have observed a diminished total body/venous hematocrit ratio averaging 0.868 in congestive heart failure, with an increase to a mean of 0.908 following cardiac compensation. The results of the present study suggest that the plasma volumes utilized in their calculations, determined by tagged albumin dilution, are overestimated by approximately 10 per cent, both before and after treatment. Recalculation of their total body/venous hematocrit ratios utilizing the revised plasma volume measurements will increase both ratios. The increase in the hematocrit ratio associated with compensation persists, however, and remains essentially of the same magnitude. If plasma volume were overestimated in the decompensated state as related to its measurement after compensation, this change in hematocrit ratio could be shown to be artefactual. However, assuming that our patient series is comparable to that of Schreiber and associates, their finding of a decreased hematocrit ratio in heart failure cannot be explained on this basis of erroneous plasma volume estimations.

Despite the quantitative differences between plasma volumes determined by RISA and dextran dilution in this study, it is notable that by either method significant plasma volume expansion was seen in nearly all patients with congestive heart failure. Application of the dextran dilution method thus confirms most previous conclusions based on albumin tagging methods concerning plasma volume changes in heart failure,[6, 19-21] although it does suggest that plasma volume has been overestimated, by approximately 10 per cent, by the latter method. Also in agreement with previous studies[6, 19-21] is the observed decrease in plasma volume associated with cardiac compensation in these patients, with the exceptions noted.

The relative unavailability of high molecular weight dextran at the present time precludes its use for routine plasma volume determinations. However, this method seems particularly applicable as a check on the validity of plasma volume measurements by tagged albumin dilution. Such comparative studies would be particularly pertinent in the various edematous clinical states, where increased flux of protein from serum to lymph may occur, and in shock, burns, or other conditions in which increased capillary permeability to macromolecular substances is present.

SUMMARY

Simultaneous plasma volume determinations with I^{131}-tagged serum albumin and high molecular weight dextran have been performed on groups of normal subjects and patients with congestive heart failure and edema, studied before and after diuresis.

In all groups studied the volume of distribution of tagged albumin was significantly greater than that of dextran. This discrepancy was greater in

patients with heart disease than in the normal subjects. No change in the discrepancy between the two methods was observed following treatment of heart failure with loss of edema.

The data suggest that albumin escapes from the circulation in larger amounts than dextran, and that this loss occurs during the period of intravascular mixing. This results in a falsely high calculated plasma volume based on the dilution of albumin. This phenomenon appears to be exaggerated in heart disease.

Previous studies showing an expansion of plasma volume in congestive heart failure have been confirmed by the dextran method.

The use of high molecular weight dextran is proposed as a check on the validity of plasma volume measurements by albumin tagging in other pertinent clinical states.

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A COMPARISON OF THE WATER DIURESIS PRODUCED BY ORAL AND BY INTRAVENOUS WATER LOADING IN NORMAL HUMAN SUBJECTS

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INTRODUCTION

METABOLIC studies frequently utilize water loading in an attempt to produce physiologic diabetes insipidus. The water is usually administered as tap water via the gastrointestinal tract or as an hexose solution intravenously. In normal subjects, these two routes are generally considered identical insofar as dilution of body fluids is concerned and equivalent in their capacity to produce a water diuresis. As discussed by Welt and Nelson,[1] however, there are expected differences in their effects on the compartments of body fluid which might influence the renal response. These authors compared intravenous and oral water loading in different recumbent normal subjects and concluded that a water diuresis could be better maintained with the intravenous route. The current studies were designed to compare the diuresis produced by orally and intravenously administered water loads in normal individuals, each subject serving as his own control on the same day.

METHODS

Ten experiments in seven adult men were performed. Three subjects were physicians in good health; four were patients with minor skin ailments. Each subject had an unrestricted breakfast one or two hours before the study began. All subjects remained recumbent except for standing to void. No smoking was permitted and no food was taken orally after breakfast except for occasional (not over three per hour) carbohydrate candies (Life Savers).

The basic plan of each experiment was as follows: After a control period of about one hour, a water load equal to 1.7 per cent of body weight was given either as tap water orally during a 20- to 30-minute period or as a 2 per cent glucose plus 2 per cent fructose solution intravenously at an initial rate of about 30 ml. per minute. The water load was then maintained so that body weight remained approximately constant at 1.7 per cent above the control value, except as noted below. Urine was collected as voided specimens approximately every 20 or 30 minutes and subjects were weighed after each voiding. During oral loading, the water required to maintain the load was ingested during 5 to 25 minutes after each voiding. During intravenous loading, the infusion rate was varied so as to maintain the desired weight as closely as possible. After water diuresis had been established and maintained at what appeared to be an approximately maximal constant rate for one to three hours, the route of water administration was changed without allowing the positive water balance to decrease. Oral water loading was used initially in three

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studies (T. F. W., 12/11/53; W. H., 12/18/53; and D. D., 12/23/53) and was stopped as soon as the intravenous infusion had commenced. Intravenous water loading was used initially in the remaining seven experiments and, in these, the transition to oral loading was made gradually, usually during a 20- to 40-minute period. In four of the latter group of studies the positive water balance was constant throughout the experiments; in three, the change to the oral route was made with a full loading volume equal to an additional 1.7 per cent of basal body weight, in order to determine whether the results would be altered by introducing a larger total volume of water into the stomach at the time of the transition to the oral load. All experiments were continued until an approximately constant maximal rate of diuresis had been sustained by the second route of administration for one to three hours.

Intravenous solutions were at room temperature; the ingested water was the same or slightly cooler. "Arterialized" blood[2] was withdrawn through an antecubital vein with minimal stasis during the control period and at the end of loading by each route.

Methods of analysis were as described in previous papers,[3] except: serum proteins were determined by the biuret reaction[4]; analyses of blood for sugar were done by a modification of the methods of Nelson[5] and Somogyi[6]; analyses of urine for sugar were done by the method of Somogyi[7]; the freezing point depressions of serum and urine were determined with a Fiske freezing point osmometer, the machine having been calibrated to read effective osmolal concentration with sodium chloride solutions at the time of each set of analyses.[8]

Calculations were as follows:

1. Free water clearance,[9] $C_{H_2O} = V \left(1 - \frac{[Osm.]_U}{[Osm.]_P} \right)$

2. Osmolal clearance,[9] $C_{Osm.} = V \left(\frac{[Osm.]_U}{[Osm.]_P} \right)$

Where V = urine volume per minute, $[Osm.]_U$ = urinary osmolal concentration, and $[Osm.]_P$ = plasma osmolal concentration

3. Change of plasma volume
4. Change of extracellular space

} After calculations based on hemoglobin concentration, hematocrit, plasma protein concentration, and serum chloride concentration described by Elkinton and Winkler[10] assuming initial plasma volume of 2.5 L. and initial extracellular space = 20 per cent body weight. Urinary excretion of chloride and blood removed by venipuncture are included in the calculations.

Statistical analyses of the data were performed with use of a Student's "t" test in which the observations for each individual study were paired. A value of "t" of probability less than 0.05 was considered necessary to establish significance ($P < 0.05$).

RESULTS

Table I shows the measured concentration of various constituents of serum and blood before loading and at the end of loading by each route for all ten experiments.

Serum Solute Concentration.—In four of seven experiments in which the positive water balance was the same by both routes, the total osmolal concentration of the serum was lowered equally by both oral and intravenous water administration. In two, it was slightly lower at the end of oral loading; in one, comparison is impossible as no determination of serum osmolality was made during intravenous loading.

TABLE I. SERUM AND BLOOD DETERMINATIONS BEFORE WATER LOADING AND AT THE END OF CONTINUOUS LOADING BY THE INTRAVENOUS AND THE ORAL ROUTES

	SERUM									BLOOD								
	TOTAL SOLUTES (mOsm./L.)			SODIUM (MEQ./L.)			TOTAL PROTEIN (GM. %)			HCT. (VOL. %)			HGB. (GM. %)			GLUCOSE (MG. %)		
SUBJECT DATE	PRE-LOAD	I.V.	ORAL	PRE-LOAD	I.V.	ORAL	PRE-LOAD	I.V.	ORAL	PRE-LOAD	I.V.	ORAL	PRE-LOAD	I.V.	ORAL	PRE-LOAD	I.V.	ORAL
T. F. W.* 12/11/53	290	273	274	142	133	138	5.95	5.50	6.00	44.2	42.1	45.1	13.9	13.2	14.5	—	167	—
W. H.* 12/18/53	290	—	270	143	132	138	5.99	5.77	6.22	42.1	41.0	42.9	13.0	13.1	13.6	—	140	75
D. D.* 12/23/53	284	269	269	136	128	134	6.83	6.52	6.54	50.1	48.0	50.0	16.5	15.5	16.6	—	185	65
T. F. W. 1/27/54	290	276	276	139	134	136	6.69	6.28	6.64	43.0	42.7	44.3	13.1	12.5	13.7	75	110	84
W. H. 2/10/54	290	280	274	145	138	137	6.62	5.77	6.27	42.0	41.9	41.9	13.7	12.8	13.7	88	128	62
R. D. 2/12/54	282	274	274	140	134	136	6.68	6.08	6.97	48.0	47.1	50.5	14.3	13.6	14.9	62	118	62
B. C. 3/17/54	282	271	264	140	135	132	6.83	6.31	6.41	40.0	38.2	38.0	13.1	12.4	12.3	80	123	81
F.† 3/19/54	281	272	267	140	135	133	6.82	6.64	6.85	42.0	40.0	46.0	14.4	13.6	14.1	60	113	71
A. S.† 4/19/54	282	268	266	135	132	133	7.06	6.77	6.64	50.0	47.5	47.5	16.4	15.4	15.6	78	98	73
B. C.† 3/15/54	280	270	259	139	135	130	7.87	6.50	6.45	45.0	40.5	39.0	14.5	12.6	12.1	79	112	77

*Oral load preceded intravenous load.

†Transition to oral route made with full loading volume of 1.7 per cent of body weight (see text).

Magnitude of Diuresis.—For purposes of comparison, the last hour of diuresis by each route of water administration has been used because, as explained under "Methods," each route was continued until the diuresis had become approximately constant for one to three hours. Table II compares the approximate last hour of water loading by each route with respect to the rate of urine flow, C_{H_2O}, $C_{Osm.}$, endogenous creatinine clearance (C_{Cr}), and total osmolal concentration. As can be seen, there were no large or uniform differences in the magnitude of diuresis achieved by the two routes, and no significant difference could be demonstrated by statistical analysis. The sequence in which the two routes were used and the volume of water ingested when oral followed intravenous loading do not appear to have influenced the results significantly.

TABLE II. URINE DETERMINATIONS DURING THE LAST HOUR OF CONTINUOUS WATER LOADING BY THE INTRAVENOUS AND ORAL ROUTES

SUBJECT DATE	FLOW (ML./MIN.)			C_{H_2O} (ML./MIN.)			$C_{Osm.}$ (ML./MIN.)			ENDOGENOUS C_{Cr} (ML./MIN.)			TOTAL SOLUTE CONCENTRATION (mOsm./L.)	
	I.V.	ORAL	I.V.–ORAL	I.V.	ORAL	I.V.–ORAL	I.V.	ORAL	I.V.–ORAL	I.V.	ORAL	I.V.–ORAL	I.V.	ORAL
T. F. W.* 12/11/53	16.0	12.7	+3.3	12.4	9.3	+3.1	3.6	3.4	+0.2	162	127	+35	62	72
W. H.* 12/18/53	21.0	20.4	+0.6	17.2†	15.9	+1.3	3.8	4.5	−0.7	190	155	+35	49	60
D. D.* 12/23/53	15.3	15.8‡	−0.5	11.7	12.6	−0.9	3.6	3.2	+0.4	174	178	− 4	63	55
T. F. W. 1/27/54	14.9	13.0	+1.9	12.1	10.3	+1.8	2.9	2.7	+0 2	145	146	− 1	55	58
W. H. 2/10/54	18.0	20.4	−2.4	15.2	16.6	−1.4	2.8	3.8	−1.0	152	154	− 2	44	51
R. D. 2/12/54	19.9	19.8	+0.1	16.3	17.5	−1.2	3.6	2.4	+1.2	158	149	+ 9	45	33
B. C. 3/17/54	13.7	11.4	+2.3	11.3	8.7	+2.6	2.5	2.6	−0.1	139	127	+12	52	62
F.§ 3/19/54	13.9	13.8	+0.1	12.0	11.6	+0.4	1.9	2.2	−0.3	122	134	−12	39	42
A. S.§ 4/19/54	16.6	14.8	+1.8	13.4	12.6	+0.8	3.2	2.3	+0.9	150	140	+10	48	41
B. C.§ 3/15/54	12.2	10.9	+1.3	9 9	8.4	+1.5	2.3	2.5	−0 2	139	160	−21	52	58
Avg.	16.2	15.3	+0.9	13.2	12.4	+0.80	3.02	2.96	+0.06	153	147	+ 6	51	53
P(t)			>0.1 <0.2			>0.1 <0.2			>0.5			>0.3 <0.4		

*Oral load preceded intravenous load.

†Calculated on an assumed serum solute concentration of 270 mOsm./L. If 260 mOsm./L. is assumed, C_{H_2O} = 17.0; if 280 mOsm./L. is used, C_{H_2O} = 17.3.

‡Next to last hour used in view of a marked (?emotionally induced) antidiuresis during the actual last hour of oral loading.

§Transition to oral route made with full loading volume of 1.7 per cent body weight (see text).

Solute Excretion.—There was no significant difference between the osmolal clearances which occurred during the last hour of loading by each route. Furthermore, there was no directional correlation between differences in osmolal clearance and the minor differences in magnitude of diuresis encountered in the individual experiments, nor could the latter be correlated with

the rate of excretion of the individually measured solutes, sodium, potassium, and chloride (data not shown). No glycosuria occurred during the diuretic phase of any experiment.

Endogenous Creatinine Clearance.—Except as noted below, there were no large (greater than 15 per cent) or consistent differences in the endogenous creatinine clearance during the last hour of each route of water loading (Table II). However, in four of ten experiments, there were impressive decreases (more than 35 ml./min.) within the first two hours after water ingestion. Since these were not followed by rebound phenomena, they do not appear to have been due to collection errors. They were not related to sudden large changes in urine flow, although in two (R. D. and B. C., 3/17/54) there was a slight concomitant decrease of the diuresis. In two studies (W. H., 2/10/54, and R. D., 2/12/54), these reductions of endogenous C_{Cr} were transient; in the other two (W. H., 12/18/53, and B. C., 3/17/54), return to previous levels had not occurred by the end of the oral loading period.

In two experiments (T. F. W., 12/11/53, and W. H., 12/18/53), the endogenous C_{Cr} was approximately 35 ml. per minute higher during the last hour of intravenous than of oral water loading, and in each study there was an accompanying augmentation of C_{H_2O} without a significant increase in $C_{Osm.}$ (Table II). In one of these (W. H.), the difference resulted from a reduction of endogenous C_{Cr} below control values during water ingestion (as already noted); in the other (T. F. W.), the difference was due to an increase in endogenous C_{Cr} above control levels during intravenous loading.

Calculated Changes of Plasma and Extracellular Volume.—Changes in plasma volume (Table III) have been estimated from calculations described

TABLE III. CALCULATED CHANGES IN PLASMA VOLUME AND CHLORIDE SPACE DURING THE LAST HOUR OF ORAL AND INTRAVENOUS WATER LOADING*

	CHANGE IN CALCULATED PLASMA VOLUME (PER CENT OF CONTROL)						CHANGE IN CALCULATED CHLORIDE SPACE (PER CENT OF CONTROL) (ASSUMING CONTROL = 20% BODY WEIGHT)	
	FROM HGB. AND HCT.		FROM SERUM PROTEINS		AVG.			
SUBJECT	ORAL	I.V.	ORAL	I.V.	ORAL	I.V.	ORAL	I.V.
T. F. W. 12/11/53	−7.6	+5.6	−2.8	+4.8	−5.2	+5.2	−0.6†	−0.6†
W. H. 12/18/53	−8.0	−2.0	−5.6	+0.8	−6.8	−0.6	0	−1.3†
D. D. 12/23/53	−2.4	+8.0	+2.4	+6.8	0	+7.4	−1.2†	+2.4
T. F. W. 1/27/54	−9.2	+3.2	−2.0	+4.4	−5.6	+3.8	+1.9	+4.0
W. H. 2/10/54	−2.8	+5.2	+2.0	+11.6	−0.4	+8.4	0	+2.7
R. D. 2/12/54	−10.4	+5.6	−6.0	+8.4	−8.2	+7.0	+0.7	+4.4
B. C. 3/17/54	+7.2	+7.6	+3.6	+6.8	+5.4	+7.2	+1.5	+3.7
Avg.	−4.7	+4.7	−1.2	+6.2	−3.0	+5.5	+0.33	+2.19
P(t)					<0.01		<0.05	>0.01

*For the seven experiments in which the water load was equal by the two routes of administration.

†These instances of apparent reduction in chloride space may be the result of a relatively large cumulative excretion of chloride during these particular studies.

under "Methods," and from these it appears that the intravenous route of water loading resulted in a larger final plasma volume than did the oral route when the water loads administered were equal. Furthermore, in five of these seven equal-load experiments, the oral route was associated with a reduction of the estimated plasma volume below control values, whereas in six of these studies the intravenous route produced an apparent expansion of plasma volume.

Changes in extracellular volume (Table III) have been calculated on the usual assumption that there are no significant shifts of chloride between extracellular and intracellular water.[10] During oral loading such calculations are not correct estimates of alterations in extracellular volume, as any chloride sequestered in the gastrointestinal tract will result in an apparent but invalid expansion of extracellular space. Despite this artifact, the calculated extracellular volume was smaller at the end of oral loading than at the end of intravenous loading in five of the seven equal-load studies. From Tables II and III it can be seen that there was no correlation between the degree of these apparent changes in the compartments of body fluid and the magnitude of diuresis in any individual experiment.

DISCUSSION

These experiments have failed to demonstrate any significant difference in the magnitude of diuresis produced by oral and by intravenous water loading. There are many factors, however, which theoretically could explain differing magnitudes of diuresis in any one experiment.

As long as the hexose remains extracellular, intravenous hexose solutions should expand extracellular volume proportionately more than intracellular volume. With oral water loading the reverse is true as a consequence of the tendency for electrolytes to diffuse from interstitial fluid into the gastrointestinal tract,[11, 12] and of the subsequent shift of water from extracellular to intracellular space. Whether or not oral loading would be expected to cause an actual decrease in extracellular fluid would depend on the volume of water ingested and the portion of it remaining in the gastrointestinal tract at any particular time. The data reported here, in agreement with those of Welt and Nelson,[1] suggest an actual reduction of plasma and extracellular volumes during oral loading in many studies, whereas, with the same water load, the intravenous route was almost uniformly associated with an apparent expansion of these fluid compartments. There is evidence to suggest that expansion of the interstitial and/or plasma volume suppresses the secretion of antidiuretic hormone (ADH),[3, 13] and conversely that their contraction (or altered distribution) may stimulate ADH production.[14, 15] An intravenous water load might therefore suppress ADH more consistently than would an equal load administered orally, particularly in subjects who were initially somewhat dehydrated. This was not the case in the experiments reported here, but it was in those of Welt and Nelson,[1] and might be one explanation of the apparent advantage of intravenous loading in their studies.

The rate of solute excretion can affect the magnitude of a water diuresis, including the component represented by the free water clearance.[16, 17] Variations of total solute excretion in the present experiments were minor and did not correlate well with such differences as were observed in urine flow or C_{H_2O}. In the study of Welt and Nelson,[1] however, the decrease in urinary solute excretion from the beginning to the end of each experiment was considerably greater in the studies utilizing oral loading than in those using the intravenous route, another factor which may explain their observations.

It is easy to conceive that changes in the rate of glomerular filtration might alter a water diuresis. If the increases in endogenous C_{Cr} during intravenous loading in two experiments indicate changes in the rate of glomerular filtration, they might well explain the slightly greater diuresis in these two instances. Of the four studies demonstrating a major decrease in the endogenous C_{Cr} shortly after the onset of water ingestion, two had a slight concomitant diminution of the diuresis, which similarly might be a manifestation of a reduction in glomerular filtration. The explanation for these changes in the endogenous C_{Cr} is not clear. If they do in fact reflect alterations in the rate of glomerular filtration, they might relate to the increases and decreases in plasma and interstitial volume already discussed.[15, 18, 19]

The possible effects on water diuresis of metabolic alterations due to the rapid infusion of an hexose solution remain unexplored, as does any difference which may result from introducing the water into the portal and systemic venous systems respectively. Any slight lowering of body temperature, a factor which was not controlled in these studies, might tend to increase water diuresis regardless of the route of administration.[20] Finally, the rate and completeness of water absorption from the gastrointestinal tract during oral loading must be an uncontrolled and, in these studies, an unmeasured variable.

SUMMARY AND CONCLUSIONS

Continuous intravenous and oral water loading have been compared in ten experiments on seven normal recumbent subjects, each subject serving as his own control on the same day.

No significant difference in the magnitude of the water diuresis resulting from the two routes of water administration was demonstrated.

Intravenous water loading almost always produced an expansion of the calculated plasma volume, whereas oral water loading frequently resulted in an actual reduction of calculated plasma volume. The intravenous route was associated with a larger calculated chloride space than was the oral route in five of seven equal-load experiments.

The minor differences in magnitude of diuresis observed in the individual experiments could not be shown to correlate with rates of solute excretion, changes in endogenous creatinine clearance, or with the calculated alterations of extracellular and plasma volumes.

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FURTHER STUDIES OF DIABETES INSIPIDUS FOLLOWING HYPOPHYSECTOMY IN MAN

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IT IS commonly assumed that the diagnosis of diabetes insipidus is apparent on clinical grounds alone and that a few laboratory tests will suffice to distinguish between it and other diseases. On the basis of our studies with patients following hypophysectomy, it is suggested that this problem may be oversimplified. We shall show that diabetes insipidus, as with so many other diseases, may embrace varying degrees of severity shading into the normal range, and indeed that minor changes in the function of the hypothalamic-pars nervosa axis may not be easy to define.

In part, some of the past difficulties are attributable to the equating of diabetes insipidus and polyuria. Although this may serve as a clinical guide, an understanding of the pathophysiology of the disease demands a more rigorous definition. Polyuria may be seen with such diverse conditions as diabetes mellitus, chronic renal disease, psychogenic polydipsia, and hypercalcemia. Conversely, diabetes insipidus, under certain circumstances, may exist although the daily water exchange is within the normal range. Thus the urine volume, per se, does not necessarily yield information about the essential defect of diabetes insipidus, namely, an inability of the kidney to elaborate a concentrated urine due to a deficiency of antidiuretic hormone (ADH). Hence, if it can be shown that the above-named conditions are not present and that the kidney is normally responsive to vasopressin, then the demonstration of a decreased capacity of the kidney to concentrate the urine implies diabetes insipidus. It is in this sense that the term will be used throughout this paper.

Because diabetes insipidus exists in mild to severe forms, it is important to employ some method for assessing the function of the ADH system.† The response to hypertonic saline[1] is difficult to quantitate and is occasionally deceptive.[2] The difficulty in quantitation is inherent in the test, as the osmotic diuresis tends to prevent attainment of maximum urine osmolarity.

The discharge of ADH under the influence of nicotine[3, 4] may provide an adequate means of determining residual function. However, the physiologic

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†The term "ADH system" will be used in place of supra-optic-pars nervosa system because of brevity and lack of implication regarding the site of origin of ADH.

stimulus to secretion of ADH is hypertonicity of body fluids. The highest urine concentrations are obtained after dehydration. It has been suggested that wide variations in glomerular filtration rate exert little effect upon the concentrating ability of the kidney in diabetes insipidus.[5] More recent studies,[6, 7] however, have related decreased glomerular filtration rate to increasing urine osmolar concentrations in the absence of ADH. Although this point must remain sub judice at present, it has been tentatively assumed that changes in urine concentration reflect the action of ADH, particularly if there are not marked concurrent changes in filtration rate. These considerations have led us to utilize a standardized dehydration test in attempting to gauge the effects of hypophysectomy on the ADH system.

METHODS

All patients had undergone hypophysectomy in an attempt to palliate metastatic cancer. In this procedure the pituitary stalk is severed and cauterized and the contents of the sella turcica removed. Patients were excluded from these studies if there was renal disease, hypercalcemia, or abnormal fluid collections. After surgery they were maintained on cortisone acetate, 37.5 mg. to 50 mg. daily. Plasma and urine osmolarities were measured cryoscopically with the Fiske osmometer.

The response to dehydration was standardized by withholding fluids for 10 hours overnight and collecting the next 5 ml. of fresh urine by catheter. This period was chosen because of its convenience and the finding that this represented sufficient water restriction to be acutely uncomfortable to most of the patients with diabetes insipidus.

In order to obtain reproducible results, it was necessary to warn the nurses not to inform the patient of the coming fluid restriction until the hour of fluid withdrawal arrived. This is important because some of these patients will make an attempt to load themselves with water in preparation for the period of water withdrawal. The patients were kept in bed throughout to avoid possible postural effects. Food was withheld during the period of fluid restriction.

RESULTS

In Fig. 1, the average daily urinary osmolar concentrations of 24 post-hypophysectomy patients are presented in a frequency diagram. These averages are derived from the values obtained from measurements of 3 to 10 daily collections at a time when the patient had fully recovered from surgery. The control group is composed of some of these patients before hypophysectomy, as well as patients hospitalized for a variety of surgical procedures. It can be seen that there is little overlap between these two groups. Of importance is the considerable number of patients with average osmolar concentrations between 250 and 350 mOsm. per liter. The specific gravities of these urines would be between 1.008 and 1.012, a range not suggestive of diabetes insipidus as classically described. It is equally evident, however, that hyposthenuria is a usual consequence of hypophysectomy.

In 15 of these patients dehydration tests were performed and urine and plasma osmolarities determined. Fig. 2 is a plot of the urine and plasma osmolarities after dehydration. In the control group the plasma osmolarity after dehydration averaged 283 mOsm. per liter ($\sigma = 2.6$), a value not significantly different from the normal plasma osmolarity of 284 mOsm. per

liter found in our laboratory. Following hypophysectomy random plasma osmolarities have been normal, but following dehydration these averaged 289 mOsm. per liter (σ = 3.9). The difference between the standard error of the

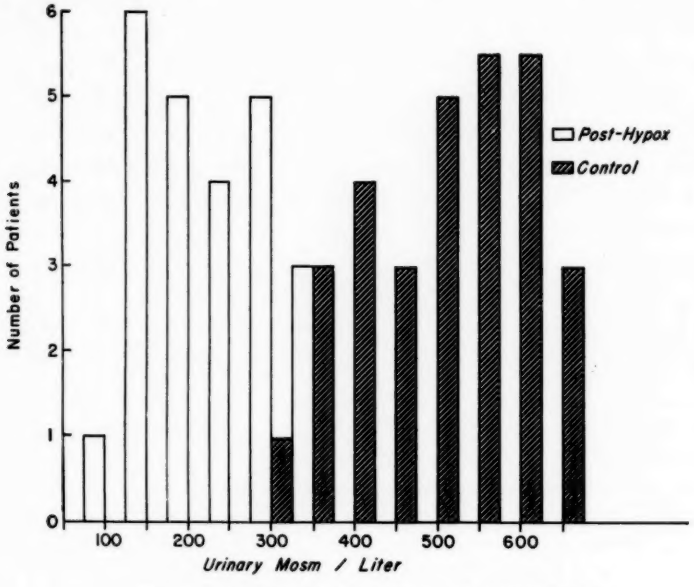

Fig. 1.—Average daily milliosmol concentration.

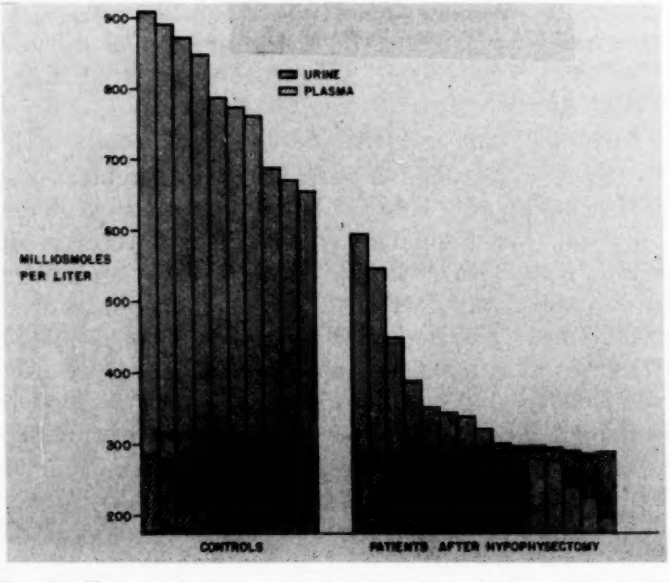

Fig. 2.—Urine and plasma osmolarities after 10 hours' dehydration.

means was 1.5, indicating that the values were significantly different. These values are tabulated in Table I. Thus, the patient after hypophysectomy can less easily defend plasma osmolarity against water loss. This is reflected in

TABLE I. URINE AND PLASMA OSMOLARITIES AFTER TEN HOURS' DEHYDRATION

CONTROLS			POSTHYPOPHYSECTOMY		
PATIENT	URINE OSMOLARITY	PLASMA OSMOLARITY	PATIENT	URINE OSMOLARITY	PLASMA OSMOLARITY
I. M.	670	284	I. M.	344	291
H. A.	890	279	H. A.	323	288
S. S.	908	286	M. R.	350	289
E. A.	847	283	R. G.	230	292
F. S.	773	280	H. A.	339	290
B. S.	761	285	F. S.	594	292
A. W.	688	284	B. S.	547	284
I. C.	786	283	A. W.	226	292
S. B.	871	288	I. C.	277	296
S. P.	654	282	M. F.	282	298
	Average 283 σ = 2.6		M. M.	449	286
			A. S.	198	289
			S. K.	386	286
			S. R.	298	287
			M. F.	301	289
				Average* 289 σ = 3.9	

*Standard error of the difference between the two means equals 1.5. Significant at 1 per cent level.

the higher plasma osmolarities. It is also evident that many of these patients are able to elaborate a urine which is hypertonic to plasma under the stress of moderate dehydration.

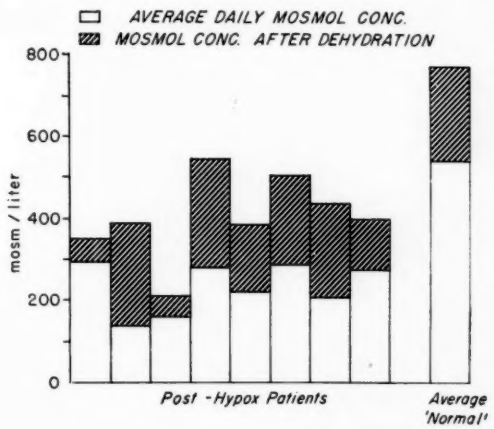

Fig. 3.—Average daily milliosmol concentration and milliosmol concentration after dehydration.

In Fig. 3 the average daily urine osmolar concentration is compared with the osmolar concentration after dehydration. All of these patients excrete urine at an osmolar concentration below the maximum available to them. Although there is remarkably little variation in urine concentration from day to day, this must represent a level of water exchange which is more comfortable than that which would be necessary for the excretion of urine at the higher osmolar concentration. The fact that these patients are capable of further concentration should make one wary of attributing changes in severity of diabetes insipidus to experimental procedures. For any regimen which

renders the patient unwilling or incapable of ingesting large amounts of fluid will lead to a rise in urinary concentration, although there have been no alterations in the endocrine milieu.

The response of 3 patients to dehydration before and after hypophysectomy is portrayed in Fig. 4. The combination of a somewhat decreased osmolar load and a relatively high urine osmolar concentration results in only slight polyuria. Clinically a diagnosis of diabetes insipidus would seldom be entertained. Yet in each case following surgery, the patient was unable to conserve water as well as before the operation. Without the preoperative tests it would be impossible to be sure that the poor response to dehydration did reflect an alteration in the ADH system. One of these patients was tested with hypertonic saline and had an equivocal response; the urine flow dropped from 7 ml. per minute to 3.2 ml. per minute, and the urine osmolarity increased from 180 mOsm. per liter to 310 mOsm. per liter at maximum antidiuresis.

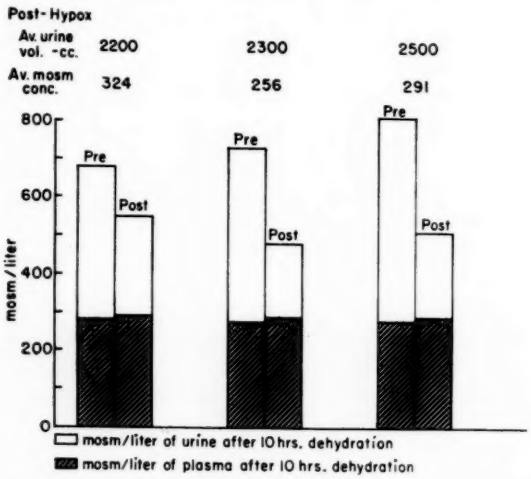

Fig. 4.—Ten-hour dehydration test pre- and posthypophysectomy.

In order to judge whether alterations in the response to dehydration are significantly different, one must have knowledge of the reproducibility of the test. In Table II are presented the results of several dehydration tests in 6 patients. No statistical analysis has been attempted, but it is apparent that the range of variation was not extreme. Thus, changes of the magnitude seen in the 3 patients in Fig. 4 probably represent real differences.

TABLE II. REPRODUCIBILITY OF TEN-HOUR DEHYDRATION TEST

	MILLIOSMOLS PER LITER AFTER DEHYDRATION											
	M.R.		R.G.		H.A.		A.W.		M.F.		I.C.	
	URINE	PLASMA	U	P	U	P	U	P	U	P	U	P
	346	287	229		307	287	227		286		314	
	384		221	292	352	283	226	289	304	297	262	
	363	291	240		310	288	224	292	251	299	255	296
	317				324		208	291	287			
							245					
Average	350		230		323		226		282		277	

The role of other pituitary factors in the genesis of diabetes insipidus is of considerable interest. Fig. 5 is a typical study of a hypophysectomized patient during a period of ACTH withdrawal. He was not receiving thyroid replacement and the protein-bound iodine was 2.1 mcg. per cent. During the period of 17-hydroxycorticoid insufficiency, the urine volumes decreased but urine osmolarities changed but slightly. The decreased urine volume reflects both a decreased food intake and a lowered glomerular filtration rate. The mild increase in urine osmolarity might have been interpreted as the result of absence of a "diuretic factor" if it had not been previously shown that this patient could reach urine osmolarity of 310 mOsm. per liter after dehydration alone. Hence, a simpler explanation of this rise in urine osmolarity is that the anorexia and nausea which accompany a lack of corticoids prevent the patient from drinking adequate fluids and, in effect, enforce mild dehydration. The decreased glomerular filtration rate may also be a factor in increasing urine osmolarity.

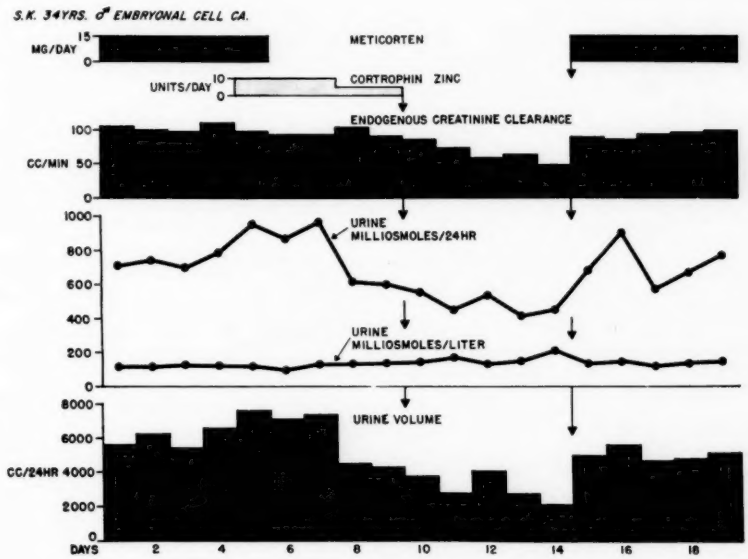

Fig. 5.—ACTH withdrawal posthypophysectomy.

DISCUSSION

A current controversy about diabetes insipidus is concerned with the site of origin of ADH. It is fortunately irrelevant to this discussion whether the pituicytes or the cells of the supraoptic nuclei are considered the source of ADH, for the operation either removes most of the pituicytes or interrupts most of the axons of the supraoptic-hypophyseal tract, leading to retrograde degeneration and a correspondingly severe loss of the cells of these nuclei. It has been claimed that there is functional reorganization of the hypophyseal stalk in the rat after stalk section.[8] Although we have been unable to test the severity of the diabetes insipidus at varying times after hypophysectomy, once established the deficiency generally persists at the same level throughout life.

Measurement of the capacity of the ADH system is admittedly difficult. Direct analyses for serum ADH are not as yet reliable.[9] Estimation of the ability of the ADH system to secrete ADH must then depend upon stimulation of the osmoreceptors and examination of the effects of the released ADH on either urine flow or concentration. As the urine flow in diabetes insipidus may vary directly with the glomerular filtration rate and pari passu with the many determinants of filtration rate, a choice of this parameter may yield unreliable information.

Knowledge of the urine osmolarity after dehydration offers a simple means of distinguishing changes in the capacity to produce ADH, particularly if preoperative studies have been made. It has been shown[10] that maximum concentration of the urine is not achieved in the normal human even with 24 hours of dehydration, so that 10 hours of dehydration will not yield an expression of the maximum capacity of the ADH system. It was hoped, however, that it would give a reproducible stimulation of the ADH system, and the results in Table II, confirm this.

It has been stated[11, 12] that diabetes insipidus is an all-or-none phenomenon, implying that the presence of any functional remnant of the ADH system would prevent the development of diabetes insipidus. This ignores certain experimental evidence[13] demonstrating that the degree of polyuria depends upon the degree of destruction of the ADH system. Two investigators[14, 15] have reported patients with diabetes insipidus who demonstrated antidiuresis in response to nicotine and dehydration. Earlier, some of us[2] suggested that diabetes insipidus could vary in severity, depending upon the degree of destruction of the ADH system resulting from surgery. A consideration of the distribution of urine concentrations in Fig. 1 again reveals considerable variation in the severity of the diabetes insipidus. The U/P ratios of Fig. 3 also tend to confirm this.

Of clinical interest is the finding that many of these patients would not give a history of polyuria if casually questioned. For example, a patient whose average urine osmolarity was 260 mOsm. per liter would need but 3 liters of urine to excrete 800 mOsm. This osmolar load is about two-thirds of the average load of the healthy person but is quite comparable to that excreted by the hospitalized or chronically ill patient. Thus, major encroachment upon the ADH system may lead to only mild diabetes insipidus, and one should not be prepared to dismiss a diagnosis of diabetes insipidus because the polyuria has not become extensive.

The role of cortisone in the expression of the polyuria of diabetes insipidus has recently been clarified. It has been demonstrated in both man[16] and animal[17] that a lack of 17-hydroxycorticoid will cause the glomerular filtration rate to fall. The fall in filtration rate in the water-loaded dog[5] can cause a drop in urine flow without change in urine osmolarity. Previous work regarding the relationship of the adrenal, thyroid, and pars anterior to the genesis of diabetes insipidus may be in error if polyuria was used as the sole criterion for assessing the effects of these glands on diabetes insipidus.

Apart from changes in renal function, cortisone increases the polyuria by increasing the appetite, thus leading to a higher osmolar load. This was clearly demonstrated by Leaf and associates[18] and confirmed by Ikkos and co-workers[19] and by us. Ikkos and associates[19] state that cortisone increased the polyuria by two mechanisms: (1) an increase in appetite and consequently osmotic load; (2) a decrease in osmolarity of the urine. This second effect would seem to be the counterpart of that mysterious "diuretic effect" of the pars anterior so often previously quoted. But as shown in Table IV from their paper, 5 of the 7 patients increased the urine osmolarity after dehydration, although it was stated that thirst tests did not induce an increase in specific gravity. Reference to our Fig. 3 reveals that these patients with the stimulus of dehydration can increase urine osmolarities above the average daily level.

With these facts in mind it is difficult to defend the thesis that cortisone decreases the osmolarity of the urine, if by this is implied some effect upon the action of ADH on the renal tubules. For in the absence of cortisone after hypophysectomy, anorexia soon ensues and the patient is unwilling to take as much fluid as before. The urine osmolarity then rises as the remnant of the ADH system is stimulated by dehydration. The fall in glomerular filtration rate which also ensues in the absence of cortisone may further increase urine osmolarity.

These points were again demonstrated in the studies shown in Fig. 5. The increase in urine osmolarity during the period of corticoid withdrawal was less than that reached after 10 hours of dehydration. The anorexia of this patient is evident in the decreased 24-hour osmolar output. The phenomena of decreased urine flow and slightly increased osmolarity may thus be seen to be due to the influence of cortisone on the appetite and glomerular filtration rate.

Ikkos and co-workers discuss one further point of theoretical interest. With use of the urine volume as an index, it is claimed that their patients exhibit a triphasic response to surgery. The classical explanation[20] of the triphasic response seen following interruption of the supraoptic-hypophyseal axis is that the initial polyuria is due to shock of the secretory cells, the stage of normal urine flow is due to release of preformed ADH, and the permanent polyuria is due to degeneration of pituicytes and neurons. This sequence of events does not occur experimentally[21] if the pars nervosa is removed, as there is no source of preformed ADH. The clinical counterpart of these animal experiments was described in man[2] when only a pituitary stalk section was performed.

The results above can be reconciled if it is realized that urine volumes alone are inadequate to establish a relationship to secretion of ADH. The initial period of polyuria is due to removal of most of the pars nervosa and shock or damage of the remaining portion of the pars nervosa. The subsequent relative oliguria may be due to return of function of this remnant, combined with the poor food intake following surgery. The third phase of polyuria may then be attributable to increased appetite and increased osmolar load.

Fig. 6 is a study of the course of diabetes insipidus in a patient following hypophysectomy. The urine osmolarities the night after surgery were 105 mOsm. per liter. Later in the postoperative period when recovery was complete, the daily average osmolar urine concentration was 210 mOsm. per liter. The initial low values represent the stage of shock to the entire ADH system; the increased urine concentration subsequently was probably due to return of function of the remnant of the ADH system.

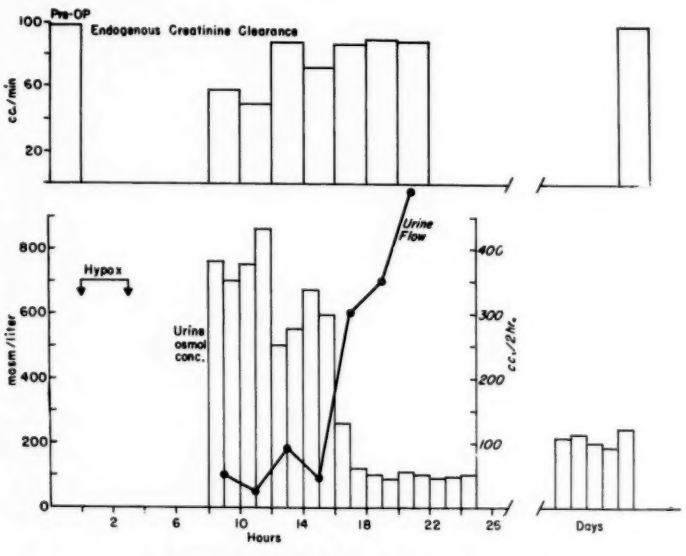

Fig. 6.—Diabetes insipidus posthypophysectomy.

SUMMARY

1. The characteristics of the diabetes insipidus resulting from hypophysectomy have been discussed. It is suggested that the severity of the diabetes insipidus depends upon the degree of destruction of the hypothalamic-hypophyseal system.

2. Considerable destruction of the supraoptic-pars nervosa system may lead to diabetes insipidus which is mild enough to escape clinical diagnosis.

3. There is no evidence that the hormones of the anterior pituitary are necessary for the production of diabetes insipidus.

4. Polyuria is but a rough clinical guide in assessing diabetes insipidus, and reliance should be placed on urine concentrations.

We wish to acknowledge the assistance of Mrs. A. Hayden.

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COPPER TRANSPORT AND EXCRETION IN NORMAL SUBJECTS AND IN PATIENTS WITH LAENNEC'S CIRRHOSIS AND WILSON'S DISEASE: A STUDY WITH CU^{64}

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WILSON'S disease (hepatolenticular degeneration) is a heredofamilial disorder characterized by an increase in the copper content of various body tissues, a decrease in plasma copper concentration, and an increase in urinary copper excretion. Aminoaciduria and hyperuricuria are also constant findings in this disease.[1, 25]

In the normal subject, plasma copper is found in association with two and perhaps more components of the plasma protein. Present evidence indicates that the major portion of this copper is bound to an alpha globulin while lesser amounts are found in the albumin and the beta and gamma globulins.[2-6] Cumings, Goodwin, and Earl[7] have reported a different distribution of copper when plasma proteins are separated by a sodium sulfate technique. The copper albumin and copper globulin fractions also have been distinguished by their ability to react with sodium diethyldithiocarbamate. Albumin-bound copper produces a direct color complex, whereas the globulin-bound copper forms the color reaction only after exposure of the plasma to dilute hydrochloric acid. These fractions have been designated as direct-reacting and indirect-reacting plasma copper fraction.[5] Markowitz and co-workers[8] in comparing the values for total serum copper, indirect-reacting copper, and ceruloplasmin in the normal subject found an excellent correlation between total plasma copper and ceruloplasmin levels. In patients with Wilson's disease there was good correlation between the indirect-reacting plasma copper and the ceruloplasmin concentration.

The hypocupremia present in these patients is attributable primarily to a decrease in ceruloplasmin concentration.[8-10, 11] Recent radiocopper studies[3, 4, 11, 14, 15] indicate that patients with Wilson's disease are unable to incorporate normal amounts of copper into ceruloplasmin. In view of this, experiments were undertaken to study the metabolism and transport of radiocopper in normal individuals and in patients with cirrhosis and Wilson's disease.

Patients with hepatic cirrhosis were included because of the intrinsic interest in the disease and in order that they might serve as controls for hepatic disease in the patients with Wilson's disease.

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METHODS

Studies were performed on four normal subjects, two patients with typical, moderately advanced Laennec's cirrhosis, and three patients with Wilson's disease. Of the latter three patients, one (M. M.) had advanced typical neurologic disease, another (W. S.) had moderately severe neurologic disease, and the third one (J. S.), who is the older brother of W. S., had no symptoms or physical abnormalities except for Kayser-Fleischer rings.

The methods for determination of total plasma copper, direct-reacting plasma copper, indirect copper, and urinary copper have been previously described.[5, 17, 18] Total plasma proteins, albumin, and globulin were determined by the biuret method.[19] Plasma bilirubin was estimated by the methods of Malloy and Evelyn.[20] Urinary amino acid nitrogen was determined according to the method of Van Slyke, MacFayden, and Hamilton.[21]

Each subject was given 3 mg. of copper as copper acetate which contained 1 mc. of Cu^{64}. Blood samples were withdrawn into heparinized, copper-free glassware at the times indicated in the various graphs. Radiocopper was determined in the blood and urine samples in a scintillation well counter. Aliquots of the homogenized pooled fecal collections were assayed for Cu^{64} in a Texas well counter. Radiocopper standards were prepared and appropriate physical decay corrections applied.

Whole plasma Cu^{64} was determined by direct count of 2 ml. samples. Plasma nonglobulin-bound Cu^{64} was measured after precipitation of globulins by the addition of an equal volume of saturated ammonium sulfate to the plasma. Globulin-bound Cu^{64} was calculated as the difference between total and nonglobulin-bound Cu^{64}.

CASE REPORTS

CASE 1.—W. S., a twenty-nine-year-old white man, was first admitted to the hospital in 1953 because of a tremor of the hands and head which had been progressive during an eighteen-month period. On physical examination, bilateral Kayser-Fleischer rings were found to be present, the liver was not palpable, and the spleen was palpable 2 cm. below the costal margin. Neurologic examination showed a gross tremor of the head and hands which was aggravated by slow movements. There was weakness and easy fatigability, but rigidity was not present. Urinalysis showed normal urine except for a persistent 2-plus proteinuria. Urine concentration tests, phenolsulfonphthalein dye excretion and intravenous pyelograms were normal. The serologic test for syphilis was negative. The chest roentgenogram was normal.

CASE 2.—J. S., a thirty-one-year-old white man, older brother of W. S., had no complaints, and has been in excellent health. Annual physical examinations necessary in his occupation had failed to reveal abnormalities. On physical examination the only abnormality seen was the presence of easily visible bilateral Kayser-Fleischer rings. Urinalysis and the serologic test for syphilis gave normal results. No abnormalities of the formed elements of the peripheral blood were found. The roentgenogram of the chest was normal.

CASE 3.—M. M., a twenty-five-year-old white woman, was first admitted to the Duke Hospital at the age of fourteen because of an upper respiratory infection. During the period from 1944 to 1955 there were four additional admissions. The diagnosis of Wilson's disease was established in 1953, at which time she complained of tremors in the left hand and foot and of an inability to coordinate her movements. She was found to have bilateral Kayser-Fleischer corneal rings, a liver palpable 2 cm. below the right costal margin, and a spleen palpable 2 cm. below the left costal margin. Urinalysis showed normal urine, serologic tests for syphilis were negative, and hematologic studies were normal except for a mild leukopenia.

Other pertinent laboratory reports are recorded in Tables I and II.

TABLE I. CLINICAL DATA ON WILSON'S DISEASE

PATIENTS*	SEX	AGE (YR.)	AGE AT ONSET	NEUROLOGIC DISORDER†	HEPATOMEGALY	SPLENOMEGALY	TOTAL PLASMA PROTEINS (GM./100 ML.)	ALBUMIN GLOBULIN RATIO	BSP RETENTION (% AT 45 MIN.)	THYMOL TURBIDITY UNITS	TOTAL PLASMA BILIRUBIN (MG./100 ML.)
M. M.	F	23	18	+++	++	+	7.7	2.1	3	1.0	0.7
W. S.	M	29	27	++	0	+	7.8	2.0	5	1.3	0.8
J. S.	M	31	–	0	0	0	7.6	1.6	2	1.3	1.2

*All patients had easily visible Kayser-Fleischer rings.
†Complete disability due to neurologic disease is equal to 4+.

TABLE II. PLASMA AND URINE COPPER STUDIES

PATIENT	TOTAL PLASMA COPPER (μG/100 ML.)	DIRECT REACTING COPPER (μG/100 ML.)	INDIRECT REACTING COPPER* (μG/100 ML.)	URINARY COPPER (μG/24 HR.)
Normal (J. B.)	93	9	84	16
Normal (E. W.)	100	7	93	42
Normal (C. G.)	100	9	91	12
Normal (F. J.)	136	20	116	17
Wilson's (M. M.)	33	22	11	258
Wilson's (W. S.)	50	42	8	625
Wilson's (J. S.)	55	51	4	151
Cirrhosis (G. S.)	123	14	109	54
Cirrhosis (W. H.)	152	8	144	72

*Calculated as the difference between the total and direct-reacting plasma copper concentration.

RESULTS

Genetic Observations.—Complete physical examinations, which included slit-lamp observations and determinations of the total plasma copper concentration, were performed on available members of the two kindreds with Wilson's disease. Genetic charts of these two kindreds are presented in Figs. 1 and 2.

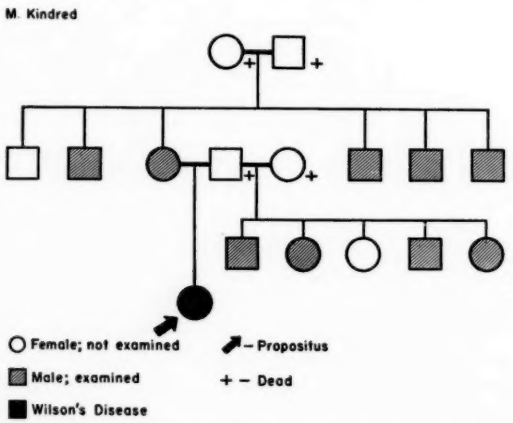

Fig. 1.—Genetic chart of the M. kindred; ten members were examined.

M. M. has no full siblings; the mother, four of five half siblings, and four of five maternal uncles were examined. All individuals were over thirty years

of age. No abnormalities referable to Wilson's disease were found, and the plasma copper levels were within the normal range.

W. S. is a member of a large family. Examination of the members of this family resulted in the diagnosis of Wilson's disease in Patient J. S. In addition, examinations were performed on eight siblings, the mother, the father, one paternal aunt, eight nieces and nephews, and the children of the affected individuals. The copper plasma levels were normal and abnormalities referable to Wilson's disease were not observed.

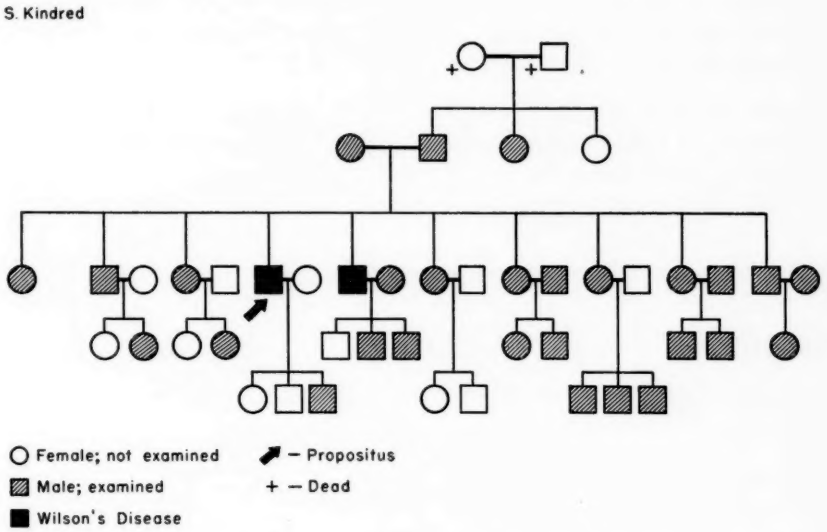

Fig. 2.—Genetic chart of the S. kindred; thirty members were examined.

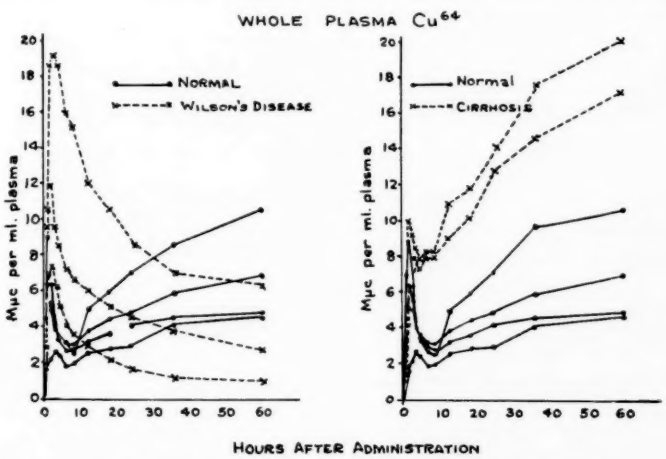

Fig. 3.—Radiocopper concentrations of whole plasma, at various intervals after the oral administration of 1 mc. of Cu^{64}, in normal subjects, in patients wtih Wilson's disease, and in patients with cirrhosis.

Plasma Radiocopper.—The difference between total plasma Cu^{64} concentration in the normal person and in the patient with Wilson's disease is depicted in Fig. 3. The initial peak of concentration was higher in two of the

patients with Wilson's disease than in the normal subjects, whereas the initial peaks of the asymptomatic patient (J. S.) and the normal subjects were comparable. In the normal subjects, an initial high point of concentration, reached in one to two hours, was followed, at six hours, by a depression to a minimum concentration. This decrease was followed by a slow but continuous rise in concentration which extended over the remaining period of observation. The early part of the curve in Wilson's disease is similar to that of the normal curve except for the markedly higher peak and the absence of the normal patients' secondary rise of concentration. Thus, despite the higher initial peak, at the end of sixty hours the patient with Wilson's disease exhibits a lower Cu^{64} plasma content than the normal subject.

Plasma Nonglobulin Radiocopper.—In all subjects, the greater portion of the initial peak of total plasma Cu^{64} was represented by nonglobulin-bound copper. The peak concentration was, however, higher in the patients with Wilson's disease than in any of the normal subjects (Fig. 4). After the peak there

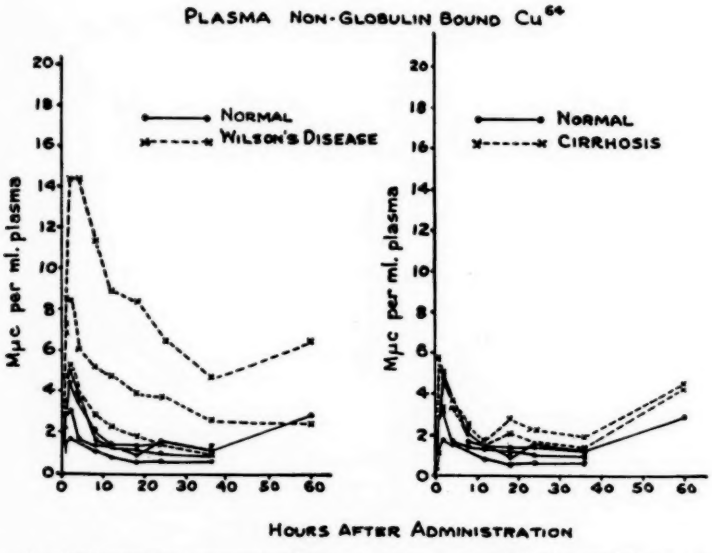

Fig. 4.—Radiocopper concentrations of plasma nonglobulin protein, at various intervals after the oral administration of 1 mc. of Cu^{64}, in normal subjects, in patients with Wilson's disease, and in patients with cirrhosis.

occurred in the normal subjects a gradual decline until a plateau was reached at twenty to twenty-four hours, whereas a plateau was not reached in the patients with Wilson's disease until approximately thirty hours after the ingestion of the Cu^{64}.

Plasma Globulin-bound Radiocopper.—The concentration of plasma globulin-bound Cu^{64} increased continuously in the normal subjects throughout the experiment. In striking contrast to this continuous increase, patients with Wilson's disease exhibited a small initial peak, coincident in time with the nonglobulin Cu^{64}, followed by a decline in Cu^{64} content (Fig. 5).

Urine and Fecal Excretion of Radiocopper.—When calculated from total plasma Cu^{64}, renal clearances of radiocopper were found to decline as total plasma Cu^{64} increased. Similar rates calculated from plasma nonglobulin-bound radiocopper, however, were reasonably constant with time. The relationship between urinary Cu^{64} and plasma nonglobulin-bound Cu^{64} is depicted in Fig. 6,

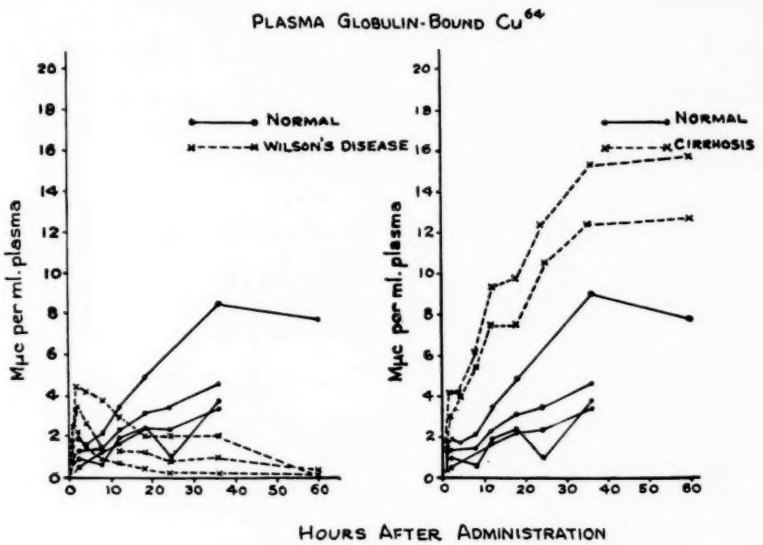

Fig. 5.—Radiocopper concentrations of the plasma globulin protein, at various intervals after the oral administration of 1 mc. of Cu^{64}, in normal subjects, in patients with Wilson's disease, and in patients with cirrhosis.

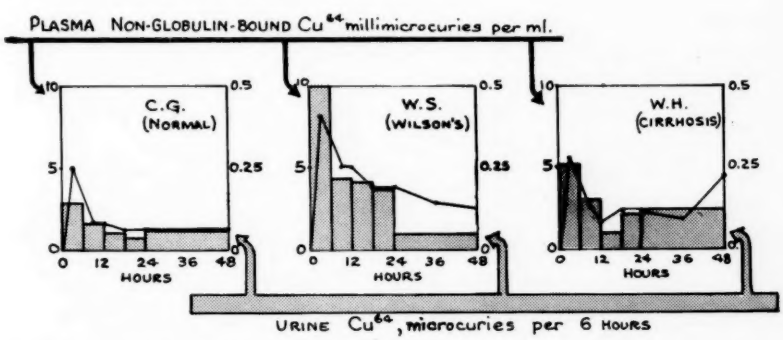

Fig. 6.—Relationship of plasma nonglobulin Cu^{64} concentration to the urinary excretion of Cu^{64}. The lines represent plasma radiocopper concentrations and the shaded areas represent urine radiocopper excretion.

and renal clearance rates calculated from plasma nonglobulin-bound Cu^{64} are presented in Table III. Clearance rates were omitted from the table during the first six hours because of the rapidly changing concentration of plasma nonglobulin-bound Cu^{64}. The renal clearance of nonglobulin-bound Cu^{64} in patients with Wilson's disease was seen to be considerably elevated when compared with that of the normal subjects.

TABLE III. RENAL CLEARANCE* OF PLASMA NONGLOBULIN-BOUND Cu^{64}

GROUPS	6-12 HR.	12-18 HR.	18-24 HR.	AVERAGE OF 6-24 HR.
Normal (J. B.)	0.226	0.204	0.198	0.209
Normal (E. W.)	0.208	0.208		0.208
Normal (C. G.)	0.301	0.245	0.149	0.232
Normal (F. J.)	0.269	0.285	0.271	0.275
Wilson's (M. M.)	0.771	(6-18 hr.)	0.959	0.834 (weighted av.)
Wilson's (W. S.)	2.330	2.620	2.740	2.560
Wilson's (J. S.)	0.446	0.527	0.671	0.548
Cirrhosis (G. S.)	0.319	0.270	0.180	0.256
Cirrhosis (W. H.)	0.404	0.132	0.335	0.290

* $\frac{UV}{B}$, milliliter/minute.

TABLE IV. EXCRETION OF Cu^{64} IN URINE AND FECES

SUBJECT	URINE Cu^{64} 0-48 HR. (PER CENT OF ORAL DOSE)	FECAL Cu^{64} 0-90 HR. (PER CENT OF ORAL DOSE)	TOTAL PER CENT OF ORAL DOSE
Normal (J. B.)	0.05	79.6	79.7
Normal (E. W.)	0.12	62.6	62.7
Normal (C. G.)	0.11	56.6	56.7
Normal (F. J.)	0.07	44.7	44.8
Wilson's M. M.)	1.82	85.3	87.1
Wilson's (W. S.)	1.65	45.5	47.2
Wilson's (J. S.)	0.22	50.0	50.2
Cirrhosis (G. S.)	0.25	51.0	51.3
Cirrhosis (W. H.)	0.20	21.8	22.0

The urine and fecal excretion studies presented in Table IV show an increase in urinary radiocopper excretion in the patients with Wilson's disease compared with the excretion in the normal subjects, but there was not a significant difference in the fecal excretion of radiocopper among the three groups of subjects studied.

Erythrocyte Radiocopper.—The concentrations of radiocopper in the plasma and red cell water are presented in Fig. 7. In the normal subjects, the plasma and erythrocyte Cu^{64} concentrations were equal at about five hours after the oral ingestion of copper, whereas in the patients with Wilson's disease, this equality did not occur until about twenty hours. The patients with cirrhosis differed from the other groups in that the concentration of radiocopper failed to attain the level observed in the plasma.

Cirrhosis.—During the first few hours the total plasma radiocopper concentration in the patients with cirrhosis was similar to that observed in the normal subjects (Fig. 3), whereas during the period from six to sixty hours, the levels of the cirrhosis patients were considerably higher. Of this increase in total plasma Cu^{64}, only a small fraction is of the nonglobulin type and the majority is found in the globulin-bound fraction. Renal clearances of nonglobulin-bound Cu^{64} were normal or slightly increased in the cirrhotic patients

(Table III). The urinary excretion of Cu^{64} was slightly increased as compared with that of the normal subjects, but still smaller than that found in the patients with Wilson's disease (Table IV).

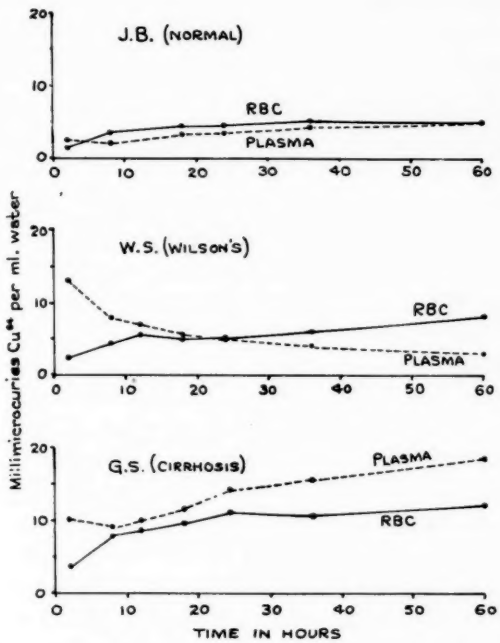

Fig. 7.—Erythrocyte and plasma radiocopper, expressed in terms of their concentrations in red cell and plasma water. Erythrocytes were assumed to have a 68 per cent water content, and plasma was assumed to have a 92 per cent water content.

DISCUSSION

Present evidence supports the hypothesis that Wilson's disease is inherited in an autosomal recessive fashion and that the affected individual inherits one gene from each parent, who, although normal, is heterozygous for the abnormal allele.[9] In accord with this hypothesis Wilson's disease would occur only in the individual who is homozygous for the abnormal allele. In this type of inheritance one would expect a ratio of affected siblings to unaffected siblings to be 1:3. In the S. family, the observed ratio was 1:4. In the M. family there were no full siblings, but the fact that the disease did not occur in five half siblings conforms to the expected pattern in a recessive type of inheritance. The failure to demonstrate abnormalities of total plasma copper levels in the parents, siblings, and offspring of the affected individuals gives further evidence that the heterozygous state is benign.

Data from other laboratories[4, 11, 15] indicate that one defect in the patient with Wilson's disease is the failure to incorporate copper in ceruloplasmin. This defect is reflected in the present studies by the absence of a secondary rise in total plasma Cu^{64} and the apparent failure of incorporation of radiocopper into the globulin fraction of the plasma proteins. Studies which were recently re-

ported by Bush and co-workers[11] have demonstrated that the early increase in globulin-bound Cu^{64}, which occurs when 50 per cent ammonium sulfate is used for separation of the plasma proteins, is not present when an immunologic technique for the determination of ceruloplasmin is employed. This early rise in globulin-bound Cu^{64} may then represent contamination of the globulin fraction by nonglobulin-bound Cu^{64}. Although patients with Wilson's disease incorporate little or no radiocopper into the globulin fraction, the presence of decreased but variable quantities of ceruloplasmin indicates that the failure to synthesize ceruloplasmin may not be absolute.

Since patients with Wilson's disease have increased plasma levels of nonglobulin-bound Cu^{64}, the proposal has been made[4] that the increased urinary excretion is a function of this elevation. The marked increase in renal clearance of the nonglobulin-bound Cu^{64} in patients with Wilson's disease indicates that the above explanation may not be adequate. This increase may be due to one or more of three possibilities: first, a difference in the physical or chemical binding of the metal by the protein, and therefore a difference in its excretion in the urine; second, an alteration in glomerular permeability which allows excess passage of copper into the glomerular filtrate; or third, an abnormality of renal tubular reabsorption whereby a small increment in plasma concentration may result in a marked increase in clearance rate. The data available do not distinguish which of these possibilities is correct. The relationship between the nonglobulin Cu^{64} level and urinary excretion of Cu^{64} suggests that the nonglobulin component is the main source of the copper found in the urine. This finding may also suggest that the nonglobulin fraction is more dissociable than the globulin-bound copper fraction. This hypothesis supports the one proposed by other investigators[4] that the metal of the nonglobulin-bound copper fraction would be readily available for tissue deposition.

The fecal excretion of radiocopper found in this study is at variance with results obtained by Matthews,[14] and by Bearn and Kunkel.[4] Bush and coworkers[11] found a decreased radiocopper fecal excretion in three of their four patients, but a normal excretion in the fourth patient. The percentage of the oral dose of radiocopper excreted by the patients reported by Bush and associates[11] was considerably greater than that reported by Bearn and Kunkel.[4] The explanation of the difference between the results found in this study and those cited above is not apparent. It should be emphasized that radiocopper excretion data under these experimental conditions are not direct measurements of total copper excretion. The dispersion of tagged copper in a larger pool of miscible body copper than there is in the normal body could conceivably alter the rate of excretion of tagged copper and perhaps explain the variant findings in the different groups of patients which have been studied.

The significance of the abnormalities in copper metabolism in patients with cirrhosis is obscure. Previous workers have described an increase in total plasma copper concentration[18] as well as in ceruloplasmin, and still other investigators have implied that the abnormally increased copper concentration may have a pathogenetic role in cirrhosis.[22] The patterns found in the two

patients with cirrhosis in this study are entirely similar to those observed in one of the two patients studied by Bush and associates.[11] In view of the hyperglobulinemia known to be associated with cirrhosis, there is the possibility that the increased globulin-bound Cu^{64} represents nonspecific binding of the radiocopper rather than increased production of ceruloplasmin. Some slight support for this was afforded by the observed late increase in the plasma nonglobulin-bound Cu^{64} and the coincident increase in urinary Cu^{64} excretion.

These data are in accord with previous studies on Wilson's disease which indicate that there is an increase in copper absorption from the intestine and, at least in certain cases, a decrease in elimination via the intestinal tract. The plasma nonglobulin-bound copper concentration is increased and the globulin-bound fraction decreased. Consequent to the high plasma levels of the more easily dissociable copper nonglobulin complex, more copper may be available for tissue deposition and urinary excretion.

SUMMARY

1. Cu^{64} was administered by mouth to four normal subjects, three patients with Wilson's disease, and two patients with Laennec's cirrhosis. At various intervals over a sixty-hour period, radiocopper concentration was measured in whole plasma, plasma nonglobulin fraction, plasma globulin fraction, red cells, urine, and feces.

2. Plasma radiocopper curves show a transient, initial rise in the nonglobulin-bound copper followed by a decline. In normal subjects, there is a secondary gradual increase in Cu^{64} which represents a continuous rise in globulin-bound copper. This secondary increase is absent in patients with Wilson's disease and is exaggerated in patients with cirrhosis.

3. The urinary excretion of Cu^{64} is closely related to the plasma nonglobulin Cu^{64}. The renal clearance, when referred to this component, is abnormally high in patients with Wilson's disease.

4. Erythrocytes appear to be permeable to plasma radiocopper.

5. Fecal excretion of radiocopper showed no marked differences among the different groups of subjects.

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PRIMARY HEMORRHAGIC DISEASES

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HEMORRHAGIC disease or a bleeding tendency may be suspected when bleeding is disproportionally greater than the trauma. Theoretically, the etiology of such pathologic hemorrhage must involve one or more of the hemostatic mechanisms normally associated with (1) blood vessels, (2) tissues (including platelets), or (3) plasma. *Primary* (congenital) defects are thought to be genetically controlled, inherent protoplasmic anomalies that give rise to a bleeding tendency usually persistent throughout life. *Secondary* (acquired) hemorrhagic tendency is associated with some underlying pathology.

Rapid recent advances in the understanding of blood coagulation mechanisms have allowed the development of many new specific tests designed to quantitatively assay the various clotting factors. Our own experience of the past few years is based upon the use of a battery of hemostatic tests applied to a large number of normal subjects and some 240 patients with bleeding symptoms. This communication presents findings in normal subjects and in 101 patients suffering from various types of primary hemorrhagic disease. Table I classifies known and postulated types of congenital disorders, with citation of selected references and inclusion of the respective numbers of each type encountered in the present investigation.

MATERIALS

Saline: 0.85 per cent NaCl. *Buffered saline:* 9 parts saline plus 1 part imidazole buffer,[24] pH: 7.3. *Thromboplastin:* Solu-Plastin (courtesy of Schieffelin & Co.). *Ca-Tpln:* Equal volumes of 0.02 M $CaCl_2$ and Solu-Plastin, mixed fresh daily and maintained at 37° C. during test periods. *Thrombin* (courtesy Parke, Davis & Co.). *Dog fibrinogen:* For prothrombin Method II: obtained from $BaSO_4$-treated (10 Gm./100 ml.) oxalated plasma by fractionation with $(NH_4)_2SO_4$ at 25 per cent saturation (25° C.), dissolving the precipitate in 1/3 original volume and dialyzing against 0.005 M sodium citrate in saline. The test preparation approximated 1 per cent clottable protein and was stored at –20° C. *Bovine fibrinogen:* Used for other two-stage procedures: 1 per cent solution of Armour's Fraction I, treated with $BaSO_4$ (1 Gm./100 ml.), stored frozen, and diluted by addition of two volumes of buffered saline before use. *Proconvertin:* Serum obtained from recalcified oxalated bovine plasma, after standing at 4° C. for 18 hours, was treated with $BaSO_4$ (10 Gm./100 ml.). After centrifugation the $BaSO_4$ was washed four times with distilled water and eluted with 1/5 the original volume of 0.2 M sodium citrate, and the eluate was dialyzed. *Accelerin*[24]: $BaCO_3$ (4 Gm./100 ml.)-treated beef serum.

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TABLE I. CLASSIFICATION OF PRIMARY HEMORRHAGIC DISEASES

ABNORMALITY	NO. CASES	REFERENCES
A. VASCULAR. *Congenital vascular purpura*		
Hereditary hemorrhagic telangiectasia	2	1, 2
Congenital angiomata	2*	
Pseudohemophilia, with spiders	3	3, 4, 5
Pseudohemophilia	5	
B. CELLULAR. *Congenital platelet purpura*		
Familial thrombocytopenia	2	6
Thrombocytopathia:		4, 7, 8
a. Lacking retractor factor	1	
b. Lacking thromboplastic factor	0	
c. Lacking accelerator factor	0	
d. Lacking serotonin (vasoconstrictor factor)	1	
C. PLASMA. *Congenital plasma purpura*		
a. Lacking AHF (hemophilia A)	52	
b. Lacking PTC (hemophilia B; Christmas disease)	26	9, 10, 11, 12
c. Lacking PTA (hemophilia C)	0	13
d. Lacking 4th factor (hemophilia D)	0	14
e. Lacking Hageman factor	0	15
f. Lacking prothrombin	0	16, 17
g. Lacking proconvertin (SPCA, Factor VII, etc.)	4	18, 19
h. Lacking proaccelerin (ACG, Factor V, etc.)	5*	20, 21
i. Lacking fibrinogen	1	22, 23

*The 2 cases of congenital hemangiomata with traumatic bleeding and 1 hypoproaccelerinemia with no bleeding history are excluded from the count (101).

Substrates: Used in specific assays of clotting factors, were prepared in large batches, divided into small lots, and stored frozen at −20° C. New lots were obtained every 4 to 6 months. "Prothrombin times" on the prothrombin, proconvertin, and proaccelerin substrates should be longer than one minute. *Prothrombin:* $BaSO_4$ (10 Gm./100 ml.)-treated bovine oxalated plasma mixed with 1/10 of its volume of proconvertin (see above). *Proconvertin:* Oxalated beef plasma passed twice, in 200 ml. lots, through Seitz filter pads containing 20 per cent asbestos (Fietrox-Werk, A.G., St. Gallen, Switzerland). Purified (proconvertin-poor) bovine prothrombin may be added to restore the prothrombin content (usually about 60 per cent), although this is not necessary. *Proaccelerin:* Normal human oxalated plasma frozen, thawed, and aged 2 to 4 weeks at 4° C. *AHF:* Citrated plasma (containing adequate platelet products) from a proved severe hemophiliac whose clotting time exceeded 60 minutes and whose prothrombin consumption was negligible. *PTC:* Citrated plasma from a proved severe case of PTC deficiency.

Standards (also stored frozen in small lots): *Standard plasma* was pooled normal oxalated plasma. *Standard $BaSO_4$-citrate eluate* was prepared from a portion of normal standard plasma by adsorbing with $BaSO_4$ (10 Gm./100 ml.) for 10 minutes at room temperature. After centrifugation the $BaSO_4$ was washed twice with saline and twice with distilled water, and was eluted with 1/3 of the plasma volume of 0.2 M sodium citrate.

COLLECTION AND PREPARATION OF BLOOD SAMPLES

Venous blood was drawn through a No. 19 or No. 20 needle into two siliconed syringes. Fifteen milliliters obtained in the first syringe was distributed: (a) 9 ml. into 1 ml. 0.1 M sodium oxalate; mixed, centrifuged 10 minutes at 2,500 r.p.m.; the plasma was removed, chilled, and used in tests 8 to 14 and 23 to 26; (b) 5 ml. into a siliconed bottle containing 10 mg. Na_2 sequestrene, which was used for venous platelet count, hematocrit, and other cell counts, and the plasma was used for fibrinogen assay. Blood from the second syringe was distributed: (c) 2 ml. samples into new glass or siliconed 13 × 100 mm. tubes for clotting times, (d) 4.5 ml. into a new glass tube for prothrombin consumption and other serum tests, and (e) 9 ml. into 1 ml. 0.1 M sodium citrate (or 1 per cent Na_2 sequestrene in 0.7 per cent NaCl) in a siliconed tube for preparation of platelet suspension. After 20

minutes' centrifugation at 800 r.p.m. (4° C.), approximately 3 ml. of platelet-rich plasma, containing only a very rare W.B.C. or R.B.C. (microscopic examination), was obtained, the volume was measured, and the plasma was recentrifuged at 20,000 g for 1 hour. The platelet-poor plasma was removed and reserved cold in a siliconed tube. The sedimented platelets were washed 6 times with saline containing 0.2 per cent Triton (WR 1330, courtesy Rohm & Haas) and finally resuspended smoothly in 1/10 volume of saline.

METHODS

The diagnostic methods employed in this study were either standard procedures (listed below) or ones which we have modified or developed in our laboratory. Each test has been numbered to simplify recording data in tables. Tests marked "*" were duplicate clotting times at 37° C. Those marked "†" were performed by our adding 0.2 ml. 0.025 M $CaCl_2$ to 0.25 ml. test mixture in a *new* glass 12 × 75 mm. tube, determining clotting time at 37° C. and, after 1 hour, adding 0.05 ml. 0.1 M sodium oxalate, winding out the clot on an applicator stick and assaying the residual "serum" prothrombin by Method I, employing a 1 to 5 dilution of the "serum."

1. *Bleeding time:* (a) Duke, (b) Ivy. 2. *Tourniquet test.* 3. *Platelet count.* The mean of two counts, one on capillary and one on venous blood, employing phase microscopy and Rees-Ecker diluting fluid. 4. *Clotting time*:* (a) glass, (b) silicone. 5. *Clot retraction:* Observed on clotting time tubes at 1 and 24 hours (37° C.) and visually graded 0 to ++++. 6. *Clot lysis:* Observed at 24 hours (37° C.) and recorded as present only if no residual clot was present. Red cells free from the clot were frequently observed and were *not* considered evidence of lysis. 7. *Prothrombin consumption:* Percentage of prothrombin disappearing in 1 hour at 37° C. Prothrombin was assayed in the serum from Tube "d," which remained undisturbed for 1 hour before oxalation with 0.05 ml. 0.1 M Na oxalate. 8. *Prothrombin time**[25]: 0.2 ml. Ca-Tpln plus 0.1 ml. plasma. 9. *Plasma-thrombin clotting time*:* 0.1 ml. thrombin, plus 0.1 ml. plasma. Thrombin concentration was regulated so that the normal time was 15 ± 1 seconds. 10. *Prothrombin:* (a) Method I, modified from Owren[26]*: 0.2 ml. Ca-Tpln plus (0.1 ml. 1 to 10 buffered saline dilution of plasma plus 0.1 prothrombin substrate); (b) Method II: The Ware and Seegers[24] two-stage method, modified by further addition of proconvertin (1 to 100) to the diluting fluid; (c) Method III: $BaSO_4$ eluate quantitatively prepared from 3 ml. plasma or serum in the same fashion as the standard eluate, and 0.1 ml. added to an incubation mixture containing 0.1 ml. accelerin (1 to 8 dilution), 0.1 ml. thromboplastin, 0.1 ml. proconvertin, 0.5 ml. imidazole buffer, and 3.6 ml. saline. At zero time 0.5 ml. 0.15 M $CaCl_2$ was added and activation was allowed to proceed for 5 minutes to a stable clotting time, obtained in triplicate by adding 0.2 ml. mixture to 0.2 ml. fibrinogen. 11. *Proconvertin*[26]*: 0.2 ml. C-Tpln plus (0.1 ml. 1 to 10 diluted plasma plus 0.1 ml. proconvertin substrate). Not more than 15 seconds must elapse between the preparing of the plasma dilution and the start of the test. 12. *Proaccelerin:* (a) Method I (similar to the method of Stefanini[27]*): 0.2 Ca-Tpln plus (0.1 ml. 1 to 10 diluted plasma plus 0.1 ml. proaccelerin substrate); (b) Method II: A 2-stage procedure which is based upon prothrombin Method III but uses standard $BaSO_4$-citrate eluate and substitutes 0.1 ml. 1 to 10 diluted fresh $BaSO_4$-treated plasma for accelerin. Clotting times were obtained at minute intervals for 10 minutes. The high plasma dilution was sufficient to prevent visible clot formation in the incubation tube. 13 and 14. *AHF*† and *PTC*† assays: 0.2 ml. standard AHF- or PTC-deficient plasma plus 0.05 ml. of various dilutions of normal and patient's plasma or serum. 15. *Fibrinogen:* gravimetric determination of fibrin, deposited on a weighed glass rod after 0.5 ml. (or more) plasma was diluted with 5.0 ml. buffered saline and clotted with 500 units of thrombin, the clot wound out, thoroughly washed with saline and distilled water, and dried (85° C.) to a constant weight. 16. *Platelet "Thromboplastin"*†*:* 0.2 ml. normal platelet-poor plasma (see "Preparation") plus 0..05 ml. (a) saline, (b) normal washed platelet in various dilutions, starting with the suspension diluted 1 to 100, or (c) patient's platelets in appropriate dilutions. 17. *Platelet accelerator:* Proaccelerin assay, Method II, was adapted by substituting 0.1 ml. full-strength platelet suspension for the 1 to 10 diluted $BaSO_4$-treated plasma. 18. *Platelet retractor factor:* The ability of

patient's and normal platelets to cause clot retraction was compared by observing, at 30-minute intervals, recalcified tubes containing 0.2 ml. normal platelet-poor plasma plus 0.05 ml. (a) normal platelet suspension, (b) patient's platelet suspension, and (c) saline. 19. *Serum vasoconstrictor factor (serotonin):* Oxalated serum specimens, from tube (d) were frozen, shipped, and assayed (M.B.Z.) by a previously published method.[28] 20. *Serum antithrombin:* 1 ml. oxalated serum was treated with 100 mg. $BaSO_4$, centrifuged clear, and mixed with an equal volume of thrombin. Clotting times were noted at 1-minute intervals after addition of 0.2 ml. of the mixture to 0.2 ml. fibrinogen. Initial thrombin concentration was adjusted so that when saline was substituted for serum, the clotting time was 15 ± 2 seconds. 21. *Serum platelet agglutinating factor* (compare 29): 0.2 ml. $BaSO_4$ serum (as for antithrombin) was mixed with 0.2 ml. normal platelet suspension (1 to 10 dilution) and was allowed to stand 2 hours at room temperature and overnight at 4° C. Gross appearance was noted at 2 hours and gross and microscopic appearance the next morning. 22. *Circulating anticoagulant*†:

MIXTURE (NO.)	1	2	3	4	5	6
Normal plasma (ml.)	0.25	0.20	0.15	0.10	0.05	0.00
Patient's plasma (ml.)	0.00	0.05	0.10	0.15	0.20	0.25

If clotting times were prolonged and prothrombin consumption decreased in Tubes 2-5, the test was considered positive. A titer was then set up using dilutions of patient's plasma in Tube 2. 23 and 24. *Anti-AHF*† and *Anti-PTC*†: AHF or PTC titers on mixtures of equal volumes of normal plasma and (a) patient's plasma or (b) saline, preincubated 30 minutes at room temperature. If the AHF or PTC content of the normal plasma was significantly reduced by incubation with the patient's plasma, a titer was set up employing dilutions of the patient's plasma. 25. *Antitissue thromboplastin**: 0.1 ml. human brain thromboplastin was substituted for Solu-Plastin in the prothrombin time test and clotting times were compared with those of normal plasma. In doubtful cases human brain and plasma were preincubated before adding calcium. 26. *Protamine titration*†: 0.2 ml. patient's plasma plus 0.05 ml. protamine sulfate of various strengths (0.005 per cent to 0.1 per cent). Test was considered positive if any strength protamine improved clotting time and prothrombin consumption.

Conversion of Observed Clotting Time to Percentage Normal.—A uniform system of stating results as per cent of normal required (a) definition of normal, i.e., the mean value observed in a large group of healthy subjects, (b) reference curves for converting clotting times of normal dilutions to per cent normal, and (c) standards of fixed value, to be assayed daily together with patients, thus controlling method variations.

At the beginning of the study, assays for the various factors (tests 8 to 17, 20) were carried out on 38 normal subjects, 8 of whom took turns throughout the study in serving as controls. The frozen standards were also tested. The mean value in each test was taken as 100 per cent. Then, for Methods 8; 10a, b, c; 11; 12a; 13; 14; and 16, five to ten samples which had the highest values (shortest clotting-times) were pooled and reassayed at precise dilutions, thus extending each reference curve from well above 100 per cent to as low as the practical limits of the methods. Only in the most exceptional cases, therefore, did an unknown require dilution to afford a value that could be read from the reference curve.

The frozen standards proved reliable for prothrombin, proconvertin, and PTC assays; they were replaced and reassayed every 3 to 4 months. Blood from one of the controls was used as a standard each day in assay of the more labile factors, assuming that these individuals showed no great daily variations in these factors. Thus a small correction factor could be introduced if necessary to control any divergence due to daily reagent or technical variation.

In Methods 12b and 17, each test contained the same amounts of prothrombin, proconvertin, thromboplastin, and calcium, and the rate at which thrombin was formed was primarily dependent upon the amount of proaccelerin or accelerator factor added. The one- to five-minute clotting times showed this rate, and it was necessary to run separate curves for each patient and the daily control in appropriate dilutions in order to express the patient's value in "percentage" of the control.

For each individual, values obtained by the three specific prothrombin methods, which were consistently in close agreeemnt, were averaged to give the prothrombin figure. Likewise, values obtained by the two proaccelerin methods were averaged.

Severity of Bleeding Tendency.—Severity of bleeding tendency was graded, on the patient's history, with use of an arbitrary scale: Mild (+): posttraumatic bleeding only, e.g., easy bruising, excessive bleeding from cuts, scratches, tooth-extractions, tonsillectomy, other surgery, and epistaxis (often noted in childhood). Moderate (++): besides the above, one annual major bleeding episode such as hemarthrosis, hematuria, hematemesis, melena, hemoptysis, severe menorrhagia, internal bleeding, etc. Severe (+++): 2 to 5 major episodes per annum. Very severe (++++): 6 or more major episodes a year.

Family Histories.—Many pedigrees were studied in connection with the cases investigated, often with the testing of numerous members of their families. Pedigree numbers have been included in this communication for easy reference to a future paper,[30] dealing with genetic aspects of primary hemorrhagic disease.

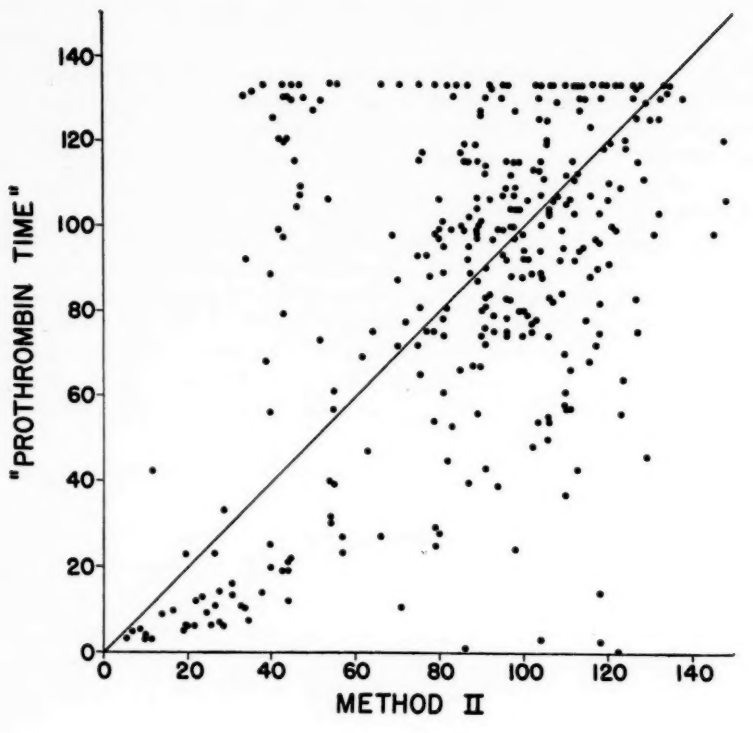
RESULTS

Normal Subjects.—Table II summarizes the test results in normal subjects (medical students, research personnel, etc.) and indicates the range of values attributable to individual variation. Results of tests on the daily controls are not included, but all were well within the recorded ranges. Fig. 1 compares graphically the data of (*A*) the prothrombin time test with specific Method II, (*B*) Method II with Method III, (*C*) Method II with Method

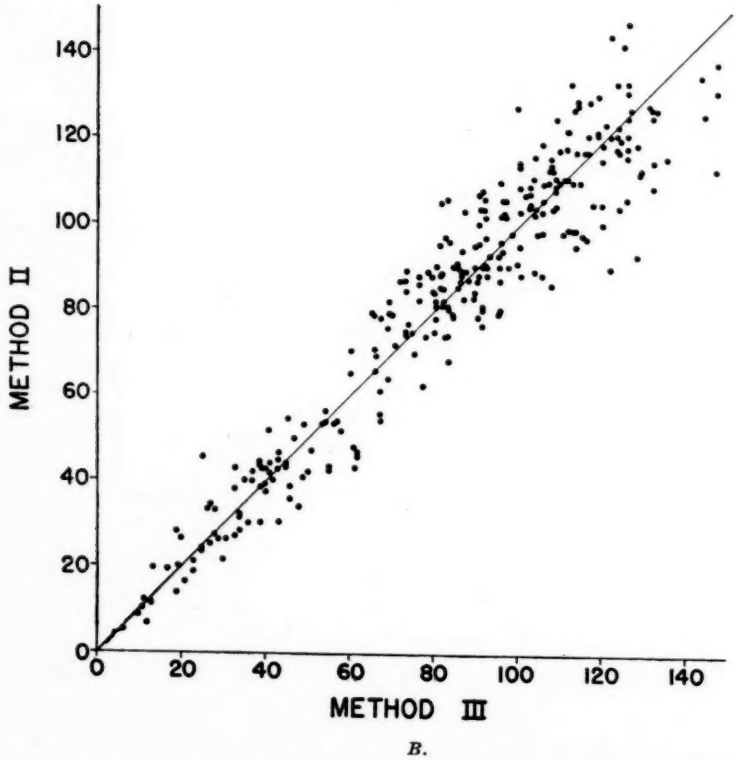
TABLE II. RESULTS OF HEMOSTATIC TEST ON NORMAL SUBJECTS

TEST	NO. OF EXAMINATIONS	MEAN	RANGE
1. Bleeding time			
Duke	58	165 sec.	60-360 sec.
Ivy	50	160 sec.	60-360 sec.
2. Tourniquet test	60	–	0-15 petechiae
3. Platelet count	48	235,000	148 to 457,000
4. Clotting time			
Glass	53	9 min.	5 to 13 min.
Silicone	53	34 min.	22 to 50 min.
5. Clot retraction	53	–	+++ to ++++
6. Clot lysis	53	0	0
7. Prothrombin consumption	60	95%	90-99%
8. Prothrombin time*	122	100%	72-133%
9. Plasma-Thr. time	53	15 sec.	13-17 sec.
10. Prothrombin			
Method I*	86	99%	65-129%
Method II*	122	99%	68-147%
Method III*	90	100%	66-146%
11. Proconvertin*	92	102%	64-149%
12. Proaccelerin			
Method I*	117	–	60-100+%
Method II*	48	–	60-100+%
13. AHF*	53	100%	70-130%
14. PTC*	53	100%	60-130%
15. Fibrinogen	50	332 mg. %	240-540 mg. % (66-148%)
16. Platelet thromboplastin*	24	100%	70-140%
17. Platelet accelerator*	24	100%	40-150%
18. Platelet retractor	24	++++	++++
19. Serotonin	29	100%†	25-150%
20. Serum antithrombin	50	–	3 min. = 40-85% thrombin 5 min. = 65-95% inactivated
21. Serum platelet agglutination	24	–	0 to ++ agglutination

*Expressed as per cent of normal.
†Equal to 0.07 mcg. of serotonin base per milliliter.

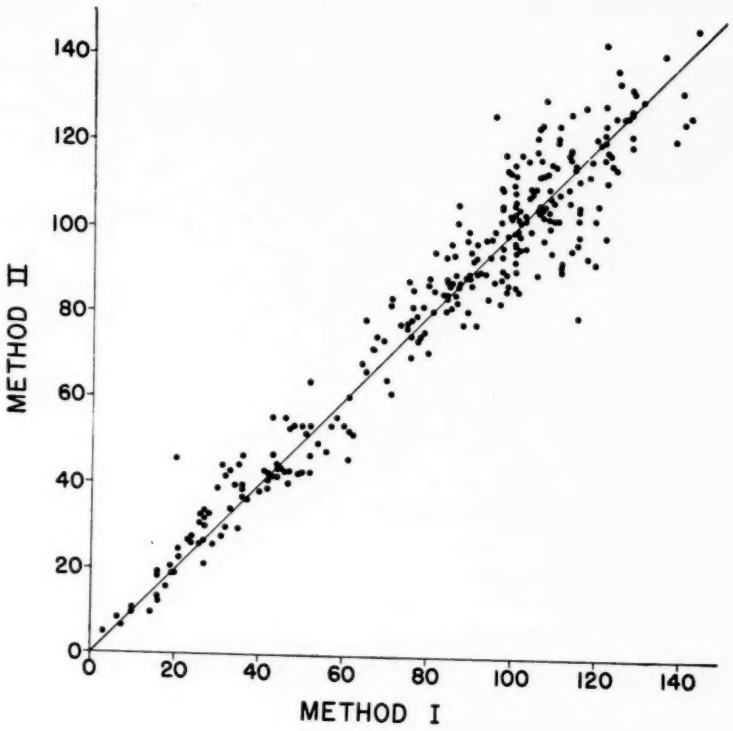


A.

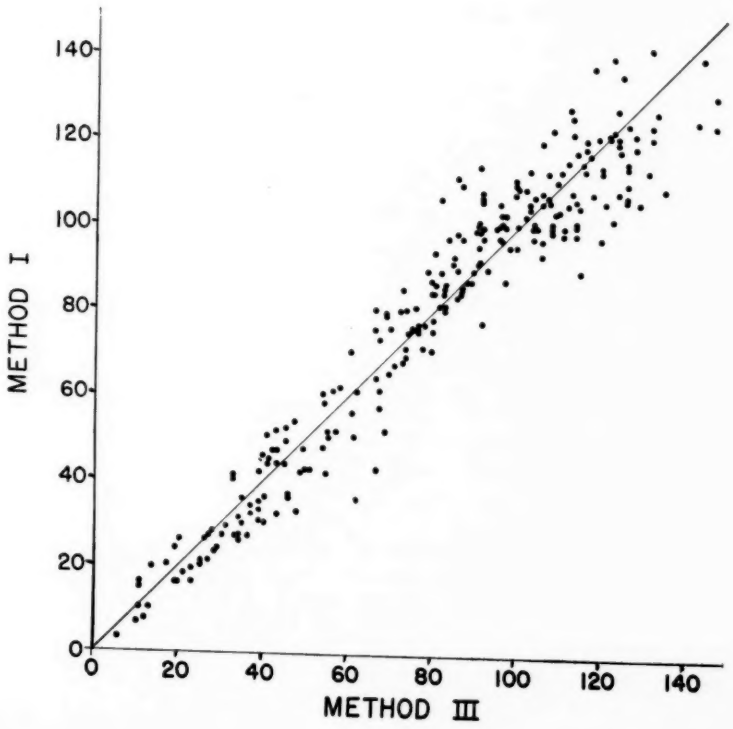


B.

Fig. 1.—*A*, *B*, *C*, and *D*, Correlation of results using three specific prothrombin assay methods and the prothrombin time.



C.



D.

(For legend, see opposite page.)

TABLE III. CONGENITAL VASCULAR PURPURA

PATIENT	NO.	AGE	SEX	RACE	F.H.	PEDIGREE NUMBER	DIAGNOSIS	AGE ONSET	PREDOMINANT SYMPTOM	BLEEDING TENDENCY	BLEEDING TIME 1A	BLEEDING TIME 1B	2 T.T.	ABNORMAL TESTS*
G. W.	83	3	F	N	0	–	Hemangiomata	2	Local	0	3	–	Neg.	None
K. P.	44	1	F	W	0	–		1	Bleeding	0	3	–	Neg.	None
Z. M.	30	43	F	W	+	–	Hereditary hemorrhagic telangiectasia	12	Epistaxis	+++	1	–	Neg.	None
P. R.	156	54	F	W	+	34		46	Hematemesis	+++	1	–	Neg.	17 = 20%
R. L.	142	34	M	W	+	37	Pseudohemophilia with "spiders"	2	Easy bruising	++	4	–	++	4b = 64 min.
S. L.	143	6	F	W	+	37		1½	Easy bruising	++	*8*	–	++	4b = 76 min.
R. C.	229	35	F	W	+	–		33	Epistaxis	++	4	–	++	13 = 50%
J. C.	22	56	M	W	+	35	Pseudohemophilia	1	Epistaxis	++	*7*	*15+*	Neg.	None
J. W.	58	17	F	W	0	–	Pseudohemophilia	8	Postoperative	+	*13*	*8*	Neg.	7 = 89%
D. Q.	150	53	F	W	+	36	Pseudohemophilia	43	Postoperative	++	*7*	*8*	+	None
A. C.	218	52	F	W	+	36	Pseudohemophilia	19	Easy bruising	+	–	*10+*	+	None
S. C.	219	20	F	W	+	36	Pseudohemophilia	6	Easy bruising	+	*7*	*10+*	++	3 = 104,000 16 = 20% 17 = 25%

*Tests 18 and 21 to 26 were omitted in all.

I, and (*D*) the latter with Method III. Data on many patients with various bleeding tendencies are included. The comparisons clearly show the good correlation ($r = > 0.93$) among the 3 specific methods and the poor correlation ($r = 0.51$) between one of them (II) and the prothrombin time test.

Hemorrhagic Diseases.—

Congenital vascular purpura: This term was selected to include those bleeding tendencies which may be due to some inherent abnormality of the vascular system. Table III lists 12 patients classified under this heading. The first two, Nos. 83 and 44, showed only local traumatic bleeding, but the blood studies were helpful in establishing the differential diagnosis between hemangioma and hematoma associated with a generalized bleeding tendency. Patients 30 and 156 were typical examples of hereditary hemorrhagic telangiectasia, giving long histories of repeated bleeding episodes and similar bleeding in the family. Typical telangiectasia, namely, localized dilatations of small tortuous purplish vessels without arterial centers, were noted on tongue and mouth surfaces of No. 30 and on tongue, face, and hands of No. 156. The laboratory studies were normal, except for an unexplained low (20 per cent) platelet accelerator level in No. 156. The remaining 8 patients, who exhibited generalized bleeding tendencies characterized by epistaxis, spontaneous bruising, and postoperative bleeding were classified under pseudohemophilia. Patients 142, 143, and 229 are subgrouped separately because they showed the additional finding of typical spider angiomata with arterial centers and radiating "legs." Unlike true telangiectasia, these spiders were not subject to rupture and local bleeding. Some evidence of diffuse, small vessel abnormality was indicated by the prolonged bleeding times and positive tourniquet tests, although neither test was consistently abnormal.

A few minimal and unexplained clotting abnormalities were also encountered. For instance, Nos. 142 and 143 showed silicone clotting times of 64 and 76 minutes, respectively; No. 229 had an AHF level of 50 per cent, and the prothrombin consumption (89 per cent) of No. 58 was just under the lower normal (90 per cent) limit. Again, No. 219 had a mild thrombocytopenia (104,000 platelets per cubic millimeter) and correspondingly reduced platelet functions (thromboplastin, 25 per cent; accelerator, 20 per cent; serotonin, 65 per cent) although her mother, No. 218, and aunt, No. 150, did not show thrombocytopenia despite as great (or greater) hemorrhagic tendency.

The family history (for details, see 30) was positive in the 2 cases with telangiectasia, and in 7 out of 8 cases (4 out of 5 families) with pseudohemophilia.

Congenital Platelet Purpura.—

Familial thrombocytopenia: Since true occurrence of this disorder is rare, our case will be described in some detail.

PATIENT A. B. (No. 41): A 2-year-old Negro boy was admitted with the chief complaints of easy bruising and petechial hemorrhages for one year. Platelet count was 38,000, clot retraction absent, and prothrombin consumption defective (70 per cent). Bleeding time was very prolonged, the tourniquet test strongly positive. Splenectomy was

performed, and a year later, there were no symptoms, platelet count was normal (320,000), and all platelet function tests were normal (with the doubtful exception of a 50 per cent accelerator. A. B.'s mother, and two maternal aunts showed normal platelet counts and function studies. The son (W. W.) of one of these aunts, now 16 years old, had suffered postcircumcision hemorrhage at 6 months, petechiae at 8 months, and easy bruising at 9 months. Bleeding symptoms continued until 6 years of age, when he was diagnosed as thrombocytopenic and splenectomized. Since then he has been free from bleeding symptoms. The son (D. D.), No. 210, of the other aunt, showed a 98,000 platelet count, positive tourniquet test, and abnormal clot retraction, platelet accelerator (10 per cent), and platelet thromboplastin (20 per cent). His sister showed a platelet count of 157,000 and moderate reduction in platelet accelerator (50 per cent) and thromboplastin (40 per cent).

Congenital Thrombocytopathic Purpura.—When the platelet count was normal but defects were found in one or more of the platelet function tests, a differential diagnosis of thrombocytopathia was made. Two examples follow:

PATIENT N. P. (No. 20): A 16-year-old white girl was admitted for study of a lifelong bleeding tendency. Prolonged vaginal bleeding occurred at time of birth, and in early childhood she had many episodes of hematomata, epistaxis, and bleeding from gums and throat. Transfusions were needed 3 to 6 times a year for the first 10 years of life, but from 10 to 13 years hemorrhagic episodes were fewer. Since menarche at 13 she has suffered severe menorrhagia and anemia, frequently requiring transfusion, the hemostatic benefits of which have been very doubtful. Hematemesis has occurred once, but melena only after epistaxis, and no hematuria or hemarthroses have been recorded. Family history, covering a rather large pedigree, notes only one possible "bleeder," a male cousin said to have suffered frequent epistaxis during childhood. Data: bleeding time—60 min.; tourniquet test—4+; platelet count—230,000; smear—platelets appear normal; clot retraction—absent. Platelet thromboplastin—100 per cent; platelet accelerator—not tested; serum serotonin—100 per cent; platelet retractor—patient's platelets would not retract a normal plasma clot, whereas normal platelets retracted her plasma clot. Tests 4, 6 to 15, and 20—normal. Capillary microscopy—normal.

PATIENT F. H. (No. 175): A 52-year-old white housewife was referred for investigation of a long-standing bleeding tendency. For the first 30 years of her life easy bruising and frequent small epistaxes were noted, but tooth extraction and menstruation gave no difficulties. However, her one pregnancy at age 29 was complicated by placenta praevia and severe hemorrhage. Menopause occurred 11 years later. In the last 20 years bruising and frequent spontaneous ecchymoses have been very marked and petechiae often appeared on the lower extremities. Melena has occurred in association with diarrhea, and hemoptysis with chest colds. No hematemesis, hematuria, or hemarthroses were recorded. Vitamins K and C have been ineffective and P (rutin), in massive doses, of questionable benefit. Careful search of the family history proved negative. Data: bleeding time—8 minutes; tourniquet test—4+, platelet count—331,000; clot retraction—normal; platelet thromboplastin—100 per cent; platelet accelerator—100 per cent; platelet retractor—normal; serum serotonin—17 per cent; tests 4, 6 to 15, and 20 to 22—normal.

Congenital Plasma Purpura.—

Hemophilia A. Fifty-two patients suffering from chronic bleeding tendencies associated with low plasma AHF levels were studied. Only one was a female, whose history is given in some detail.

PATIENT R. N. (No. 125): A 27-year-old white housewife was referred to us for evaluation of a long-standing bleeding tendency. First hemorrhagic symptom noted was bleeding on eruption of deciduous teeth. Since early childhood, frequent bruising, both spontaneous

and traumatic, excessive bleeding from cuts and scratches, frequent epistaxis, and muscular hematomata have occurred. Tonsillectomy at 14 was followed by hemorrhage and almost continuous bleeding for 4 weeks, necessitating a number of transfusions. Menstrual periods since 13 have been regular and *not* excessive, and one pregnancy at 19 was uneventful. In the last 5 years her bleeding tendency has increased. Cauterization of the cervix was followed by hemorrhage on the sixth postoperative day, lasting for 3 months and requiring 21 transfusions. Extractions of 4 wisdom teeth were followed by hemorrhages (24 hours after extractions), again necessitating transfusions. Hematuria has occurred once. Spontaneous bleeding from the gums has been frequent. Hemarthrosis, hemoptysis, and gastrointestinal bleeding have not been noted. She has received both whole blood and plasma transfusions, which have usually been associated with a temporary cessation of bleeding. The family history was not typical of hemophilia. Her father did suffer mild bleeding symptoms in his youth, but none in the past 40 years, and no other members of the family are bleeders. Both father and mother were available for study and their AHF assayed 100 per cent.

The bleeding time, tourniquet test, prothrombin time, platelet count, plasma-thrombin clotting time and assays for prothrombin, proconvertin, proaccelerin, PTC, fibrinogen, platelet accelerator, and platelet thromboplastin were normal in this patient. One-tenth milliliter of PTA-deficient plasma (kindly supplied by Dr. R. L. Rosenthal), when added to 1.9 ml. of her blood, corrected the defect in prothrombin consumption.

Positive family history was obtained in 44 of 52 hemophiliacs (85 per cent). Detailed pedigrees and blood studies in these families will be reported.[30] The racial distribution among the 52 hemophiliacs was remarkable only in the slight discrepancy between percentage of Negro hemophiliacs (19 per cent) and Negro population (26 per cent) in North Carolina. Even though some families could be traced back to pre-Civil War days, none included any evidence of interracial mixing.

Bleeding symptoms most commonly encountered in our hemophilic and PTC-deficient patients are listed in Table IV, being expressed as percentage incidence, and subdivided according to the previously described 4 grades of severity. Age at onset of hemorrhagic symptoms varied from birth, with umbilical cord bleeding, to 10 years of age, following a tooth extraction. Table IV does not distinguish symptoms according to age group. It was noted that hemarthroses and hematuria were very common in the patients over 10 years but unusual in early childhood, although hemarthrosis in one elbow was noted in a 6-month-old infant. Operations and tooth extractions resulted in hemorrhage in all patients unless they had been carefully prepared by pretransfusion.

TABLE IV. HEMOPHILIA AND PTC DEFICIENCY. PER CENT OF PATIENTS, GROUPED ACCORDING TO SEVERITY OF BLEEDING TENDENCY, SUFFRING FROM EACH COMMON SYMPTOM

SYMPTOM	ALL		MILD		MODERATE		SEVERE		VERY SEVERE	
	AHF	PTC	AHF	PTC	AHF	PTC	AHF	PTC	AHF	PTC
Easy bruising	98	96	75	100	100	89	100	100	100	100
Cuts and scratches	87	100	50	100	82	100	100	100	89	100
Hemarthrosis	67	35	0	0	64	11	78	75	74	83
Epistaxis	58	69	75	86	36	56	67	50	58	83
Gums	50	54	0	71	36	33	44	0	74	83
Hematuria	48	38	0	29	36	22	50	75	63	50
G.I.	29	15	0	0	45	11	22	50	37	17
Internal	13	11	0	0	9	0	6	25	32	33
Hemoptysis	13	11	0	0	0	0	0	25	32	33

Laboratory data on the 52 hemophilics, grouped according to severity, are given in Tables V to VIII. Low AHF levels were pathognomonic of hemophilia in the absence of anti-AHF and usually accompanied by defective prothrombin consumption and prolonged clotting time. Quantitative correlation of AHF level with severity, prothrombin consumption, or clotting time was not always demonstrated. When no trace of AHF could be detected, clotting time and prothrombin consumption were consistently abnormal. On the other hand AHF levels as low as 4 per cent (V. D., No. 137) were found in association with normal glass clotting time and normal prothrombin consumption.

The blood of 5 hemophiliacs (Nos. 12, 162, 18, 231, 174) showed the presence of an inhibitor specific to AHF (anti-AHF), presumably acquired following multiple transfusion therapy. Details concerning these patients will be published later,[31] along with an idiopathic inhibitor and two cases showing anti-PTC.

Several anomalous test values, probably unrelated to hemophilia, are noted in the tables. Low prothrombin, proconvertin, and PTC levels were found in No. 174 and may relate to his infancy. Of possible interest are the low serum serotonin levels in 5 cases (Nos. 13, 120, 114, 67, 162), which, if this were a more consistent finding, could suggest that too little thrombin was formed to release serotonin from platelets.[32] When washed platelets of two normal and two hemophilic patients (Nos. 120 and 126) were assayed, normal serotonin values (0.5 to 2.5 mcg. per 10^9 platelets) were observed. However, the washed platelets of another hemophilic (No. 72) and those of two other patients with acquired hemorrhagic disease contained very low amounts of serotonin, less than enough to account for the quantity in their sera. It is possible that the vigorous washing may have resulted in a loss of serotonin from the platelets in some cases. Unfortunately, unwashed platelets were not available for serotonin assay.

Hemophilia B or PTC Deficiency.—Data concerning 26 patients suffering from chronic hemorrhagic disease associated with low PTC levels are summarized in Tables IV, IX, and X. Two female patients (Nos. 190, 216), who belonged to a large family with many affected members, were mild clinically showing symptoms of easy bruising, bleeding from scratches, cuts, operations, and some menorrhagia. Symptomatology and familial incidence did not differ significantly from hemophilia.

Assay of PTC in the range 0 to 10 per cent is beyond the limit of accuracy of the test when our usual standard PTC-deficient patient's plasma is employed. PTC levels of 0 to 25 per cent were encountered, correlating only roughly with clinical severity, clotting time, and prothrombin consumption.

Two PTC cases (Nos. 5 and 224) showed an acquired inhibitor specific to PTC (anti-PTC) and will be discussed elsewhere. One of these, No. 5, was also studied during a period when no inhibitor could be demonstrated, and these data were chosen for inclusion in the table. A few apparently unrelated test anomalies were also noted, e.g., low serotonin in Nos. 235 and 118, prolonged bleeding time in No. 8, and a positive tourniquet test in No. 190.

TABLE V. HEMOPHILIA A—MILD (+)

NAME	NO.	AGE	SEX	RACE	F.H.	PEDIGREE NUMBER	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	13 AHF (%)	23 ANTI-AHF	OTHER ABNORMAL TESTS
T. E.	4	45	M	W	+	18	*16*	*300*	*61*	*1*	0	None
J. K.	69	67	M	W	+	11	*15½*	*150*	*80*	*8*	0	None
M. H.	226	21	M	W	+	44	*15½*	*63*	92	*30*	0	16 = 40%
B. H.	236	56	M	W	+	44	*13½*	*102*	90	*20*	0	None

TABLE VI. HEMOPHILIA A—MODERATE (++)

NAME	NO.	AGE	SEX	RACE	F.H.	PEDIGREE NUMBER	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	13 AHF (%)	23 ANTI-AHF	OTHER ABNORMAL TESTS
J. H.	79	17	M	W	+	15	*15½*	*420*	94	*5-10*	0	12 = 25%
B. H.	80	13	M	W	+	15	*14½*	*300*	91	*5-10*	0	12 = 40%
E. B.	178	32	M	W	+	18	*13½*	*86*	*77*	*0-1*	0	None
B. W.	228	34	M	N	+	13	12½	*165*	*87*	*0.6*	0	None
N. W.	144	41	M	W	+	16	12	37½	*70*	7.5	0	17 = 30%
C. R.	25	3	M	W	+	15	10½	38	*88*	*5-10*	0	None
J. D.	141	11	M	N	+	13	10½	*180*	*86*	*7.5*	0	None
V. D.	137	2	M	N	+	13	9½	*150*	91	*4*	0	None
A. W.	227	36	M	N	+	13	9½	*150*	*85*	*2.5*	0	None
G. G.	169	46	M	W	+	17	7½	*106*	*89*	*1*	0	None
A. S.	39	1	M	N	0	–	7	27	*76*	*1*	0	9 = long

TABLE VII. HEMOPHILIA A—SEVERE (+++)

NAME	NO.	AGE	SEX	RACE	F.H.	PEDIGREE NUMBER	1A CLOTTING TIME (MIN.)	1B CLOTTING TIME (MIN.)	7 PROT. CONS. (%)	13 AHF (%)	23 ANTI-AHF	OTHER ABNORMAL TESTS
R. K.	232	8	M	W	+	42	*65*	*360+*	*0*	*0*	0	None
J. K.	231	10	M	W	+	42	*57*	*360+*	*0*	*0*	+	None
R. Y.	199	28	M	W	+	6	*44*	*420+*	*3*	*0*	0	None
L. C.	34	3	M	N	+	7	*41*	*300+*	*53*	*1*	0	None
D. J.	174	½	M	W	+	8	*40+*	–	*5*	*0*	+	10 = 62%, 11 = 49%, 14 = 50%
R. M.	208	19	M	W	+	1	*37*	*420+*	*0*	*0*	0	None
T. P.	172	4	M	W	+	9	*35*	*360+*	*1*	*0*	0	None
R. K.	161	40	M	C	+	10	*29*	*240+*	*50*	*0-1*	0	1 = 9½, 3 = 151,000
T. T.	16	28	M	W	+	3	*28*	*240*	*0*	*0*	0	None
J. K.	73	51	M	W	+	12	*23*	*300+*	*46*	*1-2*	0	9 = long
N. T.	82	26	M	W	+	1	*20*	*360+*	*46*	*0-1*	0	None
C. J.	68	45	M	W	+	11	*19*	*195*	*84*	*1*	0	1 = 6+, 2 = ++
D. C.*	240	2	M	W	+	19	*14*	*120*	*4*	*0*	0	None
L. H.	112	12	M	N	+	13	*14*	*150*	*82*	*0-1*	0	17 = 10%
R. N.‡	125	27	F	W	(?)	21	*14*	*125*	*73*	*2*	0	None
W. R.*	179	23	M	W	+	14	9	*120*	*86*	*2*	0	None
C. E.*	126	11	M	W	+	11	9	*240*	*73*	*5*	0	12 = 50%
R. H.†	222	4	M	N	+	13	3	6	*83*	*1*	0	None

*Studied within 4 days of transfusion.
†Poor venepuncture.
‡Similar data were obtained on repeat examination two months later.

TABLE VIII. HEMOPHILIA A—VERY SEVERE (++++)

NAME	NO.	AGE	SEX	RACE	F. H.	PEDIGREE NUMBER	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	13 AHF (%)	23 ANTI-AHF	OTHER ABNORMAL TESTS
P. H.	28	12	M	W	0	–	*240*	*500+*	*4*	*0*	0	None
G. M.	15	17	M	W	0	–	*180*	*360+*	*0*	*0*	0	None
W. H.	11	39	M	W	+	–	*150*	–	*low*	*0*	0	None
H. C.	13	1	M	W	0	–	*102*	*180+*	*low*	*trace*	0	19 = 12%
W. B.	120	23	M	W	+	4	*90*	*240+*	*0*	*0*	0	19 = 25%
J. W.	114	7	M	W	+	1	*65*	*300+*	*2*	*0*	0	19 = 25%
F. M.	147	30	M	W	+	2	*59*	*300+*	*13*	*0*	0	None
W. T.	12	13	M	W	+	1	*57*	*300+*	*5*	*0*	0(+)‡	None
H. R.	18	19	M	W	+	5	*49*	*240+*	*0*	*0*	+	None
W. C. C.	67	28	M	W	+	1	*45*	*420*	*0*	*0*	0	19 = 25%
W. R. M.	209	38	M	W	+	2	*39*	*300+*	*0*	*0*	0	None
W. A.	238	1	M	W	0	–	*37*	*300+*	*0*	*0*	0	None
W. C.*	162	32	M	W	+	1	*28*	*180+*	*8*	*1-2*	+	19 = 25%
R. T.	72	9	M	W	+	1	*24*	*480+*	*19*	*0-1*	0	None
T. G.	9	40	M	W	+	–	*24*	*240*	–	*0*	0	None
W. McK.	132	3	M	W	0	20	*17+*	–	*3*	*0*	0	None
D. P.*	171	1	M	W	+	9	13	*85*	*42*	*1-2*	0	None
A. S.*	234	2	M	N	+	43	6	*90*	*58*	*0.2*	0	None
C. C.*†	45	1	M	W	0	–	3	3	*52*	*trace*	0	None

*Studied within 4 days of transfusion.
†Poor venepuncture.
‡Six months after initial tests.

TABLE IX. HEMOPHILIA B (PTC DEFICIENCY)—MILD (+) AND MODERATE (++)

NAME	NO.	AGE	SEX	RACE	F. H.	PEDIGREE NUMBER	SEVERITY	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	14 PTC (%)	24 ANTI-PTC	OTHER ABNORMAL TESTS
R. A.	38	4	M	W	0	30	++	*17*	*100*	*65*	*<10*	0	None
C. H.	64	19	M	W	+	27	++	6	*480*	*71*	*0*	0	None
J. H.	146	22	M	W	+	27	++	8	52	*81*	*trace*	0	None
H. H.	145	17	M	W	+	27	++	8	36	*77*	*0*	0	None
H. A.	183	17	M	W	+	26	++	13	*140*	92	*15*	0	None
W. H.	220	5	M	W	+	28	++	*18*	*94*	*88*	*<10*	0	None
L. M.	215	67	M	W	+	28	++	10	41	90	*<10*	0	None
M. H.	190	30	F	W	+	28	+	10	42	95	*25*	0	2 = +++
J. H.	189	7	M	W	+	28	+	8	52	95	*<10*	0	None
R. H.	191	10	M	W	+	28	+	7	49	95	*25*	0	None
B. H.	188	8	M	W	+	28	+	6	42	93	*<10*	0	None
C. M.	213	61	M	W	+	28	+	7	37	93	*20*	0	None
C. M.	214	63	M	W	+	28	+	7	45	92	*20*	0	None
N. M.	216	35	F	W	+	28	+	9	30	*80*	*20*	0	None
H. W.	180	22	M	W	+	25	+	7	*78*	*87*	*15*	0	None
R. W.	181	28	M	W	+	25	+	10	24	*89*	*15*	0	None

TABLE X. HEMOPHILIA B (PTC DEFICIENCY)—SEVERE

NAME	NO.	AGE	SEX	RACE	F. H.	PEDIGREE NUMBER	SEVERITY	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	14 PTC (%)	24 ANTI-PTC	OTHER ABNORMAL TESTS
W. B.	224	11	M	W	+	23	++++	*480+*	*480+*	*2*	*0*	+	22. = +
D. B.	223	9	M	W	+	23	++++	*420*	*480+*	*0*	*0*	0	None
W. S.	133	33	M	W	+	23	++++	*300*	*480+*	*0*	*0*	0	None
E. W.	8	4	M	N	+	22	++++	*360*	*480+*	*0*	*0*	0	1. = 10 min.
S. T.*	184	2	M	N	0	29	++++	*48*	–	*32*	*trace*	0	None
A. Y.*	237	9	M	W	0	45	++++	11	*360*	95	*trace*	0	None
R. J.	5	18	M	N	0	–	+++	*270*	*270+*	*2*	0	0(+)†	None (22 = +)†
D. B.	235	1	M	N	0	41	+++	*180*	*360+*	*2*	0	0	19 = 25%
Q. G.	3	40	M	N	+	24	+++	*24*	*78*	*low*	0	–	None
K. S.	118	58	M	W	+	25	+++	*23*	*120*	*39*	*<10*	0	19 = 30%

*Studied within 4 days of transfusions.
†Test values observed 8 months previously.

TABLE XI. HYPOPROACCELERINEMIA

NAME	NO.	AGE	SEX	RACE	F. H.	8 PROT. T. (%)	12 PRO A (%)	11 PRO C (%)	10 PRO T (%)	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)	17 PLAT. ACC. (%)
D. H.	17	3	F	W	0	*1*	*0*	108	112	*39*	*210*	*43*	*5*
J. H.	123	15	M	N	+	*50*	*50*	105	112	8	*60*	96	*25*
W. A.	42	29	M	W	0	*50*	*50*	101	100	13½	30	*88*	100
J. M.	54	9	M	W	0	*43*	*50*	100	109	10½	*100*	97	50
P. H.	59	5	F	W	0	*61*	*34*	90	110	10½	*90*	94	100

Other tests normal in all patients.

TABLE XII. HYPOPROCONVERTINEMIA

NAME	NO.	AGE	SEX	RACE	F. H.	8 PROT. T. (%)	11 PRO C (%)	10 PRO T (%)	12 PRO A (%)	CLOTTING TIME (MIN.) 1A	CLOTTING TIME (MIN.) 1B	7 PROT. CONS. (%)
R. S.	2	29	M	W	+	*2*	*0*	105	100+	*88*	*120+*	*59*
M. T.	1	14	F	W	+	*1*	*0*	84	90	*28*	*120*	*79*
B. C.	139	6	M	W	+	*61*	*42*	83	95	8	38	94
M. C.	140	35	F	H	+	82	*52*	92	100+	5	31	90

Other tests normal in all patients.

Hypoproaccelerinemia.—Data on 5 patients with this as the probable primary deficiency are given in Table XI. Details concerning the one really severe case, No. 17, have been published.[21] Her sister, No. 59, showed a low proaccelerin level but no bleeding tendency. The three other patients showed mild bleeding tendencies (epistaxis, easy bruising, and postoperative bleeding). One, No. 123, gave a possible familial history, as 4 of his 9 siblings were subject to epistaxis.

The prothrombin times were reduced in all and seemed to reflect the proaccelerin levels. Clotting times, particularly in silicone, were prolonged in four and prothrombin consumption was deficient in two. Platelet accelerator levels were low in two.

Hypoproconvertinemia.—Two patients, Nos. 1 and 2, previously described in detail[19] suffered severe bleeding tendency associated with undetectable levels of proconvertin prolonged prothrombin times, prolonged clotting times, and deficient prothrombin consumption. A maternal aunt of Patient 1 (No. 140) and her son (No. 139) also gave histories of mild bleeding tendency and were found to have moderate decreases in proconvertin and prolonged prothrombin times (Table XII).

Afibrinogenemia.—Our one case (No. 10) of this rare malady has been described previously.[23] He was a 3½-year-old white boy with a severe hemorrhagic tendency. There was no detectable fibrinogen, the blood did not clot on addition of strong thrombin, nor was any precipitate formed when the plasma was heated to 56° C. Tests dependent upon the presence of fibrinogen: clotting time, clot retraction, and prothrombin time, were abnormal but after a number of transfusions became normal. All other tests, including prothrombin consumption and platelet functions, were normal.

DISCUSSION

As far as we are aware, this is the first large series of normal subjects and hemorrhagic patients in which actual levels of many different coagulation factors have been determined. A wide normal range, approximately 60 to 140 per cent, was found for each factor. Repeated observations on the group of normal controls showed that daily variations in prothrombin, proconvertin, and PTC were minimal, whereas variations in fibrinogen and platelet count were wide. Such day to day variations in AHF, proaccelerin, and platelet factors could not be assessed, as no fixed standards were employed.

In most of the 101 bleeders, the detection of a single deficiency with or without associated abnormalities in general blood coagulability tests allowed a definite diagnosis. In the absence of visible lesions (e.g., telangiectasia), vascular defects were diagnosed with somewhat less certainty. The term "pseudohemophilia," although unsatisfactory, has been used for a defect, probably primarily vascular, in which a prolonged bleeding time is the one simply demonstrated anomaly. It has been pointed out[29] that the cases of V. Willebrand[3] and Jurgens[4] probably included some with primary vascular and others with platelet defects. Classically the signs and symptoms in

pseudohemophilia show marked variability from individual to individual and, in the same individual, from time to time. It is possible that the same mechanisms produce prolonged bleeding times and positive tourniquet tests in pseudohemophilia, thrombocytopathia, and thrombocytopenia. The development of thrombocytopenia in patients previously exhibiting only signs of vascular purpura has been reported.[33] Our Patient 219 may be of this type. A final differentiation of these points must await a clearer understanding of the mechanisms producing these abnormalities. Clearly, serotonin cannot be implicated in all patients, as the serum levels were normal in our series as well as in others previously reported.[34, 35]

The term "thrombocytopathia" was reserved for those patients in whom some platelet abnormality could be clearly defined. There has been no previous report of a patient like No. 175 in whom the only abnormality was a low serotonin level, although other thrombocytopathies have also shown low levels.[34] Patient 20 lacked another platelet factor, that which causes clot retraction.

Plasma coagulation factor deficiencies are readily diagnosed by the specific tests for the individual factors. Even minor depressions of proaccelerin (Nos. 123, 42, and 54) and proconvertin (Nos. 139 and 140) appear to produce mild bleeding symptoms when present as single, congenital deficiencies. This is certainly not the case in the short-term acquired lack of proconvertin associated with Dicumarol ingestion. Although our experience covers the majority of known primary clotting disorders, we have not yet encountered any case in which the possibility of PTA deficiency, fourth factor lack, or true hypoprothrombinemia could be suspected. Presumably the first two factors could be assayed in test systems similar to those described for AHF and PTC.

Nonspecific tests such as clotting time, prothrombin consumption, and prothrombin time are of great value in demonstrating the presence of a coagulation abnormality. However, in a few cases, all of these tests were normal, but titer for specific factor revealed a definite deficiency. This, again, emphasizes the value of specific titers and the need for additional substrates suitable for PTA, etc., titration.

CONCLUSIONS

A study of 240 patients with bleeding tendencies showed that 101 suffered from primary or congenital disease. Genetic studies will be published elsewhere. The present communication summarizes clinical symptomatology and evaluates a battery of test procedures which allowed classification of the patients into the following groups: *congenital vascular purpura*—10 patients (2 with hereditary hemorrhagic telangiectasia, 8 with pseudohemophilia); *congenital platelet purpura*—4 patients (2 with thrombocytopenia and 2 with thrombocytopathia); and *congenital plasma purpura*—87 patients (52 hemophiliacs, 26 PTC-deficients, 4 hypoproaccelerinemics, 4 hypoproconvertinemics, and 1 afibrinogenemic).

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THE SELECTIVE DESTRUCTION OF TRANSFUSED "COMPATIBLE" NORMAL RED CELLS IN TWO PATIENTS WITH SPLENOMEGALY

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MEASUREMENTS of red cell survival have become an integral part of clinical investigation of the pathologic physiology of the anemias. Data concerning the rates at which red cells are removed from the circulation in various situations have received intensive mathematical scrutiny,[1-5] and efforts have been made to quantitate total daily red cell destruction and production by the use of transfused red cell survival studies.[6, 7] The method most widely used to measure red cell survival in vivo is indirect and involves the separation of homologous transfused red cells from the recipient's cells by differential agglutination (the Ashby method)[8] or by hemolysis[9] of the latter. The validity of these techniques in clinical studies of extracorpuscular factors influencing red cell survival rests upon the assumption that the transfused red cells survive as do the recipient's own cells. On the other hand, the demonstration that Cr^{51} in the form of $Na_2Cr^{51}O_4$ firmly labels the hemoglobin of viable red cells,[10] and that such Cr^{51}-labeled red cells may be employed in red cell survival studies,[11] provides a direct method for the study of the survival of autogenous red cells and so involves no such assumption. Unfortunately, the use of Cr^{51}-labeled red cells has failed to replace satisfactorily the Ashby method for long-term quantitative studies because of two shortcomings: a substantial "elution" of Cr^{51} from the red cells[11] at a rate which cannot be inferred in any individual instance; and the potentially deleterious influence on red cell metabolism and survival of the Cr^{51}-globin bond, because the quantity of Cr^{51} employed in red cell labeling far exceeds tracer amounts. Alterations in red cells treated with $Na_2Cr^{51}O_4$ include a change in the electrophoretic mobility of Cr^{51}-labeled hemoglobin, impairment of red cell glycolysis in vitro, and the appearance of methemoglobin when sufficient $Na_2Cr^{51}O_4$ is present.[12] These alterations are least important in the presence of rapid red cell destruction, but limit the usefulness of Cr^{51} for long-term studies of red cell survival.

In the Ashby method, although the possibility has always existed that differences in the size, shape, and viscosity of the red cells of donors and of

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recipients might serve as a basis for selective destruction of the donor's cells by extracorpuscular mechanisms, the possibility of antigenic activity of the transfused red cells has been the most serious cause for concern in evaluating the rate of their destruction. For this reason after selection of donor red cells without ABO or Rh incompatibility it is usual to perform an indirect Coombs test on the donor's red cells after their incubation in the serum of the recipient. This procedure should be repeated several weeks after completion of the survival studies. Additional safeguards against artefacts in the Ashby method have been emphasized by various laboratories. These include preliminary counting of the inagglutinable red cells in mixtures of various known proportions of blood from donor and recipient,[5] and the simultaneous transfusion of two antigenically different but compatible populations of normal red cells.[13] Occult isosensitization may also be excluded by demonstrating that donor red cells which have been transfused a second time to the same recipient are not destroyed more rapidly than in the first transfusion.[14]

That such precautions may not always be adequate is indicated by studies presented here on two patients in whom normal transfused red cells were selectively removed from the circulation despite their serologic compatibility as it is presently defined by in vitro observations. Several interesting physiologic observations and considerations emerged from these studies.

METHOD

The red cell counts, blood hemoglobin and hematocrit determinations, reticulocyte counts, morphologic examinations of the peripheral blood and bone marrow aspirates, and liver function tests were performed by standard techniques.[15] Plasma and urine hemoglobin levels were determined by the benzidine method of Bing and Baker,[16] adapted for the use of a photoelectric colorimeter. Quantitative determinations of urobilinogen output were made from consecutive two- or four-day stool collections and twenty-four hour urine collections, employing the method of Schwartz, Sborov, and Watson.[17]

A search was conducted for autoantibodies active at 4°, 25°, and 37° C. at pH levels ranging from 6.0 to 8.0. Trypsinized Group O red cells[18] were also incubated with various dilutions of the patients' sera. The direct Coombs (antiglobulin) test[19] and the indirect Coombs test were performed with serial dilutions of several lots of antihuman-serum rabbit serum. Preparations consisting of buffy coat smears of heparinized venous blood which had been allowed to stand for one hour at room temperature were examined for the presence of "LE cells." Preparations designed to elicit erythrophagocytosis were made by the method of Zinkham and Diamond.[20]

The Ashby technique of differential agglutination was employed as modified by Ebert and Emerson[21] for the use of dried rabbit antihuman blood group serum.* Total red cell counts were performed along with the inagglutinable red cell counts. The inagglutinable red cell counts of various mixtures of red cells from the donors and of whole blood from the recipients compared well in all instances with the expected values. Following transfusion the "zero-time" inagglutinable red cell count was determined by extrapolation from the best apparent curve drawn through the actual inagglutinable counts plotted on rectilinear graph paper. For the purpose of comparison, each inagglutinable count is depicted graphically as a percentage of the zero-time value.

Sodium chromate containing Cr^{51} was employed in labeling red cells by the method described by Ebaugh and his associates[11] for studies of red cell survival. In this

*Generously supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

procedure 50 to 100 μc of $Na_2Cr^{51}O_4$ were inserted into transfusion bottles containing about 500 ml. of homologous blood or into sterile flasks containing about 50 ml. of autogenous blood. Acid-citrate-dextrose mixture was used as the anticoagulant. In all instances in which Cr^{51}-labeled homologous red cell survival was determined in these patients the same aliquot of donor cells was employed for both the Cr^{51} and the Ashby techniques. The containers were agitated intermittently at room temperature for one hour, at which time 125 mg. of ascorbic acid was added in order to reduce extracorpuscular chromium to the trivalent form.[22] After rapid infusion of the Cr^{51}-labeled blood, venous blood specimens were drawn into oxalate and hemolyzed by freezing and thawing. The Cr^{51} activity of the whole hemolyzed blood samples was determined in a well-type scintillation counter. Radioactivity was expressed in terms of whole blood uncorrected for the low levels of plasma activity observed during the first twenty-four to forty-eight hours after the injection of labeled red cells. As with the Ashby procedure, the radioactivity of each specimen is represented graphically as a percentage of the calculated "zero-time" peripheral blood activity.

Following the injection of Cr^{51}-labeled red cells, body surface counts were performed with a directional scintillation counter which was positioned in contact with, and at right angles to, the skin over the center of the surface projections of the heart, liver, spleen, and sacrum of each patient. Since it was desirable to correct body surface counts for the changing Cr^{51} activity of the blood (i.e., to express a crude organ: blood ratio), body surface counts are presented as the ratio of the activity over the designated organ to that of the precordium at the same time.[12]

CASE REPORTS

Case 1.—This patient was a thirty-nine-year-old white female chronic alcoholic who entered the hospital with complaints of weakness and leg pains. The patient had had two normal deliveries seventeen and nineteen years previously. She had never been transfused. On physical examination a large liver extended 10 cm. below the anterior right costal margin, and on several occasions the spleen tip was palpable. There was slight sensory loss over both feet. Laboratory studies indicated mild impairment of liver function. Liver biopsy revealed marked fatty metamorphosis, alcoholic hyalin, and early cirrhosis. During the period of observation there was no evidence of blood loss. On admission the red cell count was 2.6 million per cubic millimeter, the hemoglobin 7.8 Gm. per cent, and the hematocrit 26.9 per cent. The mean corpuscular volume (M.C.V.) was 103 cu. μ and the mean corpuscular hemoglobin concentration (M.C.H.C.) was 29 per cent. Attending spontaneously increasing hemoglobin levels, reticulocyte counts ranged from 5 to 10 per cent for over three weeks. The patient's white cells appeared normal. Bone marrow examination revealed moderate erythroid hyperplasia. Attempts to elicit evidence of red cell sensitization or circulating antibodies were unsuccessful, except for the detection transiently during early hospitalization of a weak panagglutinin, most active at 4° C. Direct and indirect Coombs tests were consistently negative. Several buffy coat "LE cell" preparations were negative. Diagnoses included Laennec's cirrhosis with associated anemia and peripheral neuritis.

The patient, whose blood type was BMN cDE, was transfused successively by five normal donors of the following blood types: OMN cDe, OM cDe, ON cDE, OMN CDe, and OMN CDe.*

Case 2.—This patient was a fifty-year-old white housewife who entered the hospital because of massive splenomegaly. Thirteen years previously the patient had been told that she had an enlarged spleen and polycythemia. Since that time she had been treated with x-ray to the spleen, radioactive phosphorus, nitrogen mustard, and venesection. In the

*The Ashby survival data in this patient were excluded from a previously reported study of a group of patients with cirrhosis and anemia[23] because of the findings indicating incompatibility with normal red cells reported herein. Characteristically, however, patients with cirrhosis and anemia manifest diminished survival by either the Ashby method using homologous red cells[23, 24] or by the Cr^{51} method employing autogenous red cells.[12, 23, 25]

year prior to the studies reported here the patient had been given a course of x-ray to the spleen and had had 5,500 ml. of blood removed by venesection. The patient had been pregnant ten times and had had nine normal children. She had never been transfused.

Physical examination at the time of these studies revealed a firm, nontender, and massively enlarged spleen filling the left upper and lower quadrants of the abdomen and extending into the right lower quadrant, and an enlarged liver extending 4 cm. below the anterior right costal margin.

The red cell count was 6.3 million per cubic millimeter, the hemoglobin was 11.8 Gm. per cent, and the hematocrit 44.3 per cent. The M.C.V. was 70 cu. μ and the M.C.H.C. 27 per cent. Reticulocytes ranged from 1.2 to 3.3 per cent. The white cell count was 32,400 per cubic millimeter with 50 per cent adult neutrophils, 39 per cent band neutrophils, 1 per cent eosinophils, 6 per cent basophils, 2 per cent myelocytes, and 2 per cent lymphocytes. Occasional nucleated red cells were seen in the peripheral blood. The platelet count was 437,000 per cubic millimeter. Examination of bone marrow aspirates revealed a normal erythrocyte to granulocyte ratio. Hyperplasia of the erythroid and myeloid elements was noted in a section of the sternal marrow obtained by surgical biopsy. The results of liver function tests were normal. The blood Hinton test was variably and weakly positive, although the patient denied previous luetic infection or recent exposure. No autoagglutinins or autohemolysins active at 25° or 37° C. could be demonstrated in the serum. A cold panagglutinin active at 4° C. was present in a titer of 1:64. The Coombs test was negative. An "LE cell" preparation of the peripheral blood was negative. The diagnosis was made of polycythemia vera with myeloid metaplasia of the spleen, and superimposed iron deficiency caused by repeated venesections. It is recognized that the first syndrome is frequently an early manifestation of chronic myelogenous leukemia.

The patient, whose blood type was Group OMN CDe, was transfused successively with blood from normal male donors with the following blood types: ON CDe, ON cDe, and ON CDe.

RESULTS

The survival curves of normal homologous red cells in these two patients are depicted in Figs. 1 and 2. Autogenous red cell survival is indicated by square symbols. Empty symbols indicate measurements by the Ashby technique and solid symbols those by the Cr^{51} technique. It is evident that in each patient, despite the almost normal survival of autogenous Cr^{51}-labeled red cells observed during the same period of study, an abrupt drop occurred in the level of circulating red cells from the first donor about two weeks after transfusion, according to the Ashby technique.

In Case 1 the survival of the red cells of Donor 1 paralleled autogenous red cell survival for the first eleven days after transfusion (Fig. 1). However, on the seventeenth day, it was observed that inagglutinable red cells had completely disappeared from the patient's blood. Transfused red cells from Donors 2, 3, and 4 were cleared from the circulation of this patient without any initial delay, so that half the transfused red cells disappeared within one to two days. The administration of 200 mg. of cortisone daily, orally, did not appear to improve the survival of red cells from Donor 4. However, in remarkable contrast to the red cells from the first four donors, those from Donor 5 survived as well as the patient's red cells as measured on the same sample of blood by both the Ashby and the Cr^{51} techniques. Later, red cells from Donor 4 were given to this patient a second time and were again rapidly destroyed.

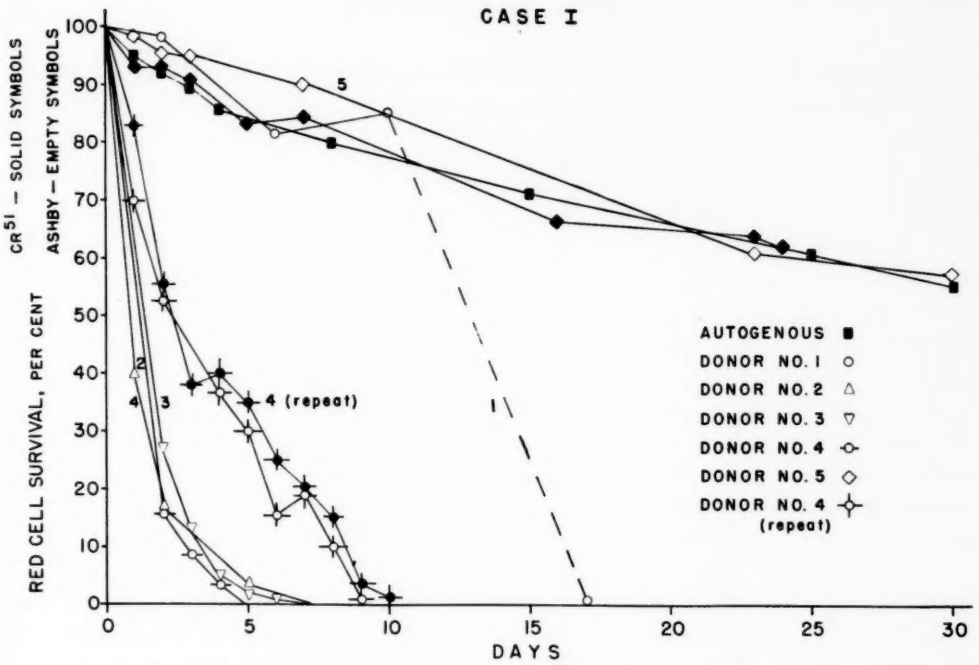

Fig. 1.—The survival, in Case 1, of red cells from five normal donors, as measured by the Ashby method (empty symbols) and by the Cr^{51} method (solid symbols). The patient's own Cr^{51}-labeled red cells (solid squares) survived normally in terms of this method (half-survival time: 33 days). The red cells of Donor 1 survived normally for at least eleven days and then abruptly disappeared from the circulation. Except for the red cells of Donor 5, which survived normally, those of subsequent donors underwent rapid removal from the beginning, as measured by either method.

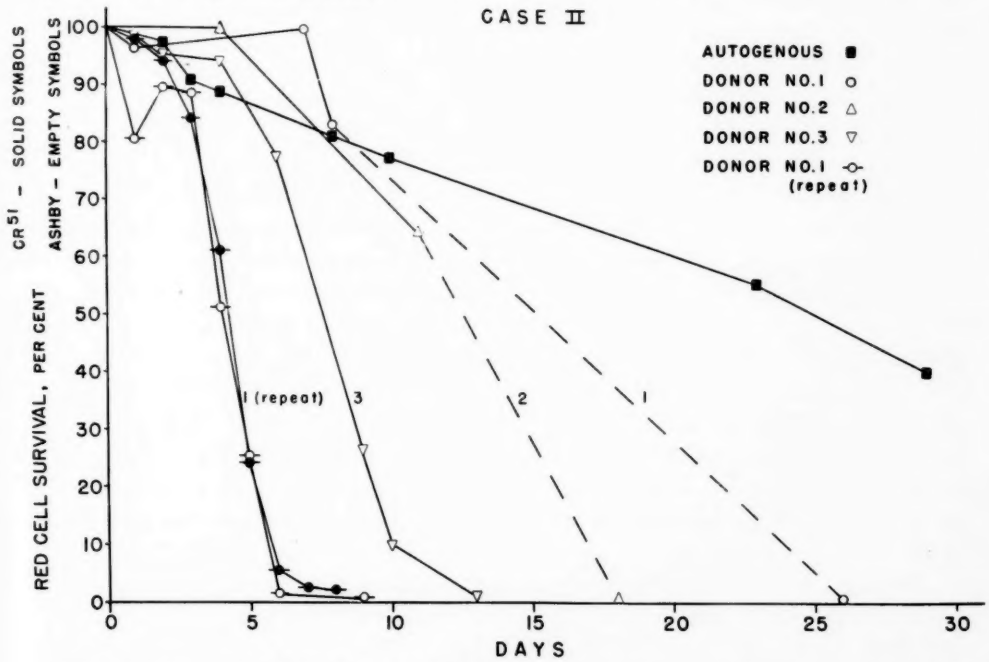

Fig. 2.—The survival, in Case 2, of red cells from three normal donors as measured by the Ashby method (empty symbols) and by the Cr^{51} method (solid symbols). The patient's own Cr^{51}-labeled red cells (solid squares) survived almost normally (half-survival time: 25 days). The red cells of all three donors survived normally for from two to ten days before a period of rapid red cell destruction set in, producing sigmoidal survival curves. An acceleration of the destructive process with each successive transfusion is evident.

In Case 2 the survival of normal transfused red cells grew increasingly brief with each transfusion (Fig. 2). In contrast to the results in Case 1 with Donors 2, 3, and 4, the short red cell survival curves in Case 2 are sigmoidally shaped as a result of a period of several days of normal survival that in each instance preceded a rapid phase of red cell removal.

In studies of the blood of each recipient, the red cells of every donor were found to be compatible in vitro with the respective patient's serum, both before and after transfusion. In each case prolonged incubation of the various donors' packed cells and the recipient's serum failed to sensitize the donors' cells as determined by the Coombs test or by suspension of the cells in 20 per cent bovine albumin. Efforts to elicit unusually weak sensitization by incubating the donors' cells at various levels of pH and temperature, with various titers of patient's serum diluted both with saline and with 20 per cent albumin, were unsuccessful. Trypsinization of the red cells of Donors 3 and 4 did not lead to agglutination in the presence of the first patient's serum. Five per cent buffered polyvinylpyrrolidone[26] failed to reveal sensitizing antibodies in the first patient's serum against the red cells of Donors 3 and 4. At the conclusion of these studies neither patient's serum contained antibodies against the known blood group antigens.*

In Case 1 there was no evidence of an abnormal clinical response to any of the transfusions. For four days following the fourth transfusion, hemoglobin determinations on individual urine specimens showed no hemoglobin, and the plasma hemoglobin level remained normal after this transfusion. No rise in icterus index followed any of the transfusions. In Case 2 an immediate mild urticarial reaction attended the first transfusion, and an immediate urticarial reaction, followed six hours later by a febrile reaction, without elevation of plasma hemoglobin, appeared after the fourth transfusion. There was no reaction to the other transfusions. Neither hemoglobinuria nor a rise in the icterus index appeared after any transfusion. No erythrophagocytosis of transfused red cells was demonstrable in the peripheral blood of either patient.

Simultaneously with studies of their in vivo survival in Case 2, washed red cells from Donor 4 were incubated in vitro with defibrinated whole blood from the patient in a concentration of about 20 per cent of the total red cell count of the mixture. After sterile incubation for eight days the donor's inagglutinable red cell count had fallen 15 per cent from the original level, whereas the recipient's agglutinable cell count had fallen 6 per cent. Thus, there was little difference in the survival of the donor's red cells in vitro compared to those of the patient. Meanwhile, however, as portrayed in Fig. 3, 90 per cent of the transfused red cells of Donor 4 had been destroyed in vivo within eight days.

In Figs. 3 and 4 are depicted the body surface radioactivity measurements following the transfusion of Cr^{51}-labeled red cells from Donor 4 into Case 1 and from Donor 2 into Case 2, respectively. The radioactivity of the spleen and liver areas, relative to that of the precordium, is plotted against time.

*A search for antibodies against red cell antigens C, Cw, c, D, E, e, Fy^a, Jk^a,Jk^b, K, k, Le^a, Le^b, Lu^a, M, N, P, S, and s was kindly performed in both cases by Dr. A. R. Jones of the Blood Grouping Laboratory, Boston, Mass.

Although the number of points for Case 1 is small, both patients show a rise in apparent spleen activity and to a lesser extent in liver activity, which developed coincidentally with a fall in the whole blood Cr^{51} activity.

Although the total urobilinogen excretion was measured in Case 1 for eleven days following transfusion of blood from Donor 4, daily excretions remained below 60 mg. (Fig. 3). In Case 2, fecal urobilinogen excretion was

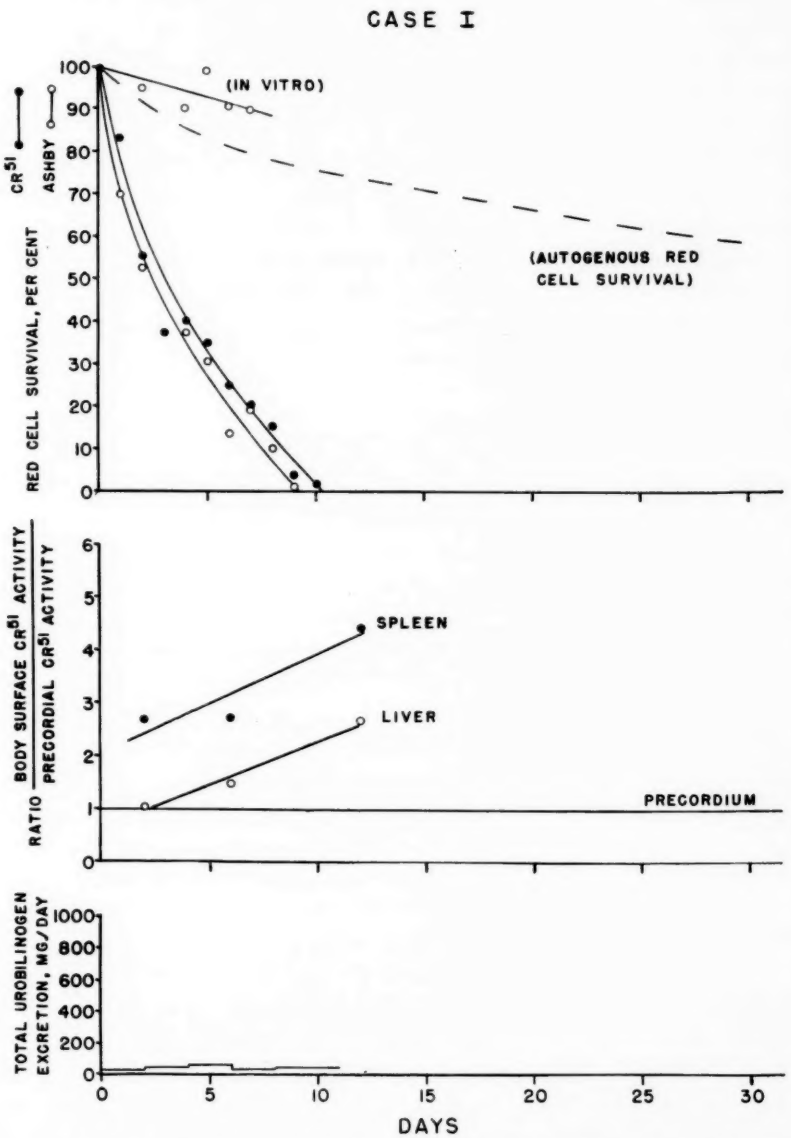

Fig. 3.—The fate of normal serologically compatible red cells from Donor 4 in Case 1. In the upper portion of the figure is evident the rapid ("exponential") destruction of these red cells in vivo by both the Ashby (empty symbols) and the Cr^{51} (solid symbols) techniques, whereas this donor's red cells incubated in the patient's whole blood in vitro survived well. In the midportion of this figure the high splenic uptake of Cr^{51} derived from the labeled donor red cells is depicted, indicating splenic sequestration. In the lower portion of the figure the combined stool and urine urobilinogen levels fail to reflect the destruction of 500 ml. of transfused blood within eleven days.

followed for a longer period after transfusion from Donor 1. During the third four-day period following transfusion, the daily excretion of urobilinogen rose sharply to 1,035 mg. from the average base line value of 250 mg. per day. The maximum excretion of 1,230 mg. per day was reached during the fourth four-day period after transfusion (Fig. 4).

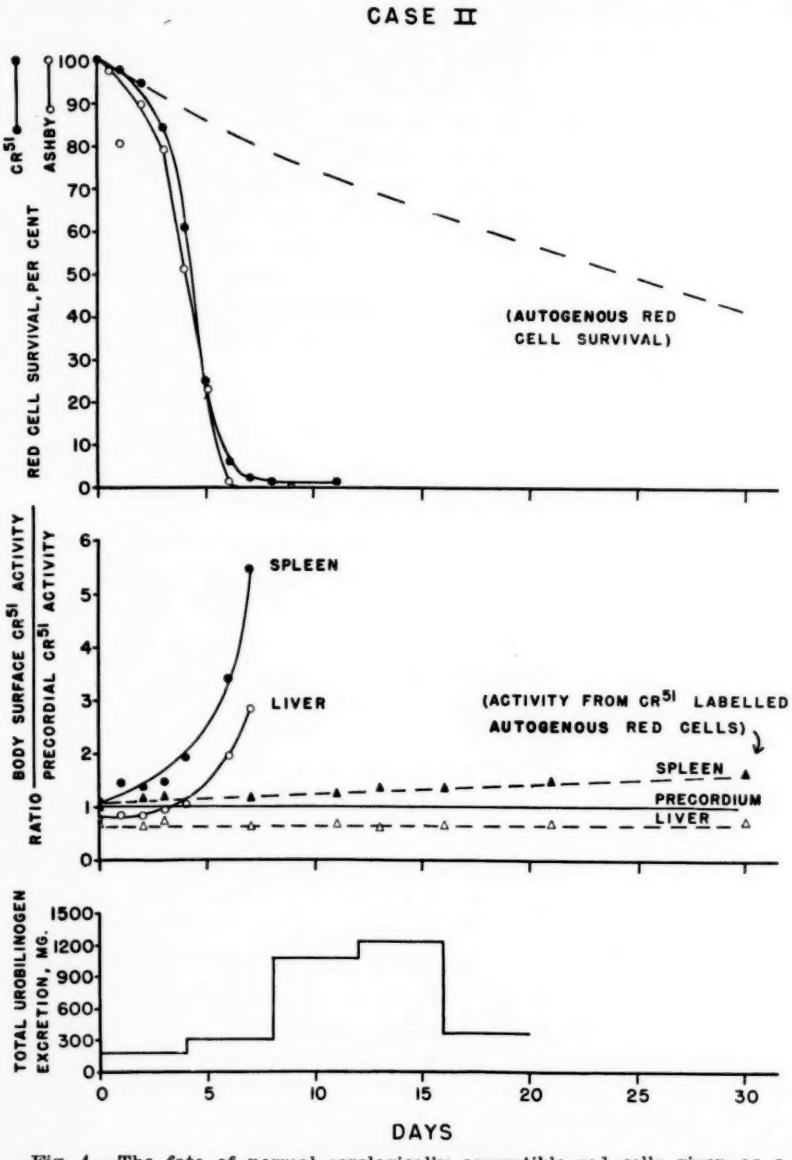

Fig. 4.—The fate of normal serologically compatible red cells given as a repeat transfusion from Donor 1 in Case 2. In the upper portion of the figure, sigmoidal red cell survival curves by both the Cr^{51} and Ashby techniques indicate the initially delayed but later rapid destruction of these red cells in the patient. In the midportion of the figure the abrupt uptake by the spleen, and, to a lesser extent, by the liver, of Cr^{51} from the transfused cells is shown (circles). This contrasts with the slow accumulation by these organs of Cr^{51} derived from the patient's own labeled red cells (triangles). Whereas the rate of uptake of Cr^{51} from autogenous red cells was far slower, the relative uptakes by spleen and liver were similar to those of the red cells of Donor 1. Below is depicted the marked subsequent increase in urobilinogen excretion, the mid-point of which followed the mid-point of red cell sequestration by about eight days.

DISCUSSION

Despite the absence of incompatibility demonstrable in vitro between the red cells of any of the donors and the respective sera of the two recipients, the selective removal of transfused red cells from the circulation observed in vivo had many of the characteristics of an immune process. Thus, in both patients, the red cells of the first transfusion appeared to survive as well as autogenous cells for about two weeks, after which the inagglutinable counts fell abruptly. In both patients, red cells from subsequent transfusions were destroyed more rapidly; and in Case 2 the rate of destruction increased with each transfusion. Also consistent with a specific immune process was the normal survival in Case 1 of red cells from Donor 5, whereas the red cells from a subsequent transfusion from Donor 4 were again rapidly destroyed.

Although measurable differences in M.C.V. and M.C.H.C. between donor and recipient red cells existed, the red cells of neither recipient possessed striking morphologic peculiarities at the time of the transfusions. The similarity of the survival curves of transfused cells from the same donor, when measured simultaneously by the Ashby and by the Cr^{51} methods, indicates that a serologic artefact in counting inagglutinable red cells was not responsible for the findings. Furthermore, in Case 1, the red cells from Donor 4 while suspended in vitro at 37° C. in the whole blood of the patient remained intact and unagglutinated for as long as eight days. In vitro studies also revealed that the accuracy of the inagglutinable red cell counts was not altered by changes in the ratio of inagglutinable to agglutinable cells.

It thus appears that an immune process selectively removing the normal red cells of certain other persons was present in both patients although serologic incompatibility in vitro was not demonstrable by presently known techniques. Theoretically this immune process may have involved either a fixed tissue antibody or a weak or qualitatively peculiar circulating antibody. The failure to demonstrate phagocytosis in vivo or in vitro of the donor red cells by the patient's white cells suggests that the leukocytes, at least, did not participate in a tissue immune process. On the other hand, a similarity is clear between the sequence of events in Case 2 and in the experimental studies reported by Swisher and Young[27] in which a dog, given repeated transfusions of canine red cells of a different blood type, destroyed each successive population of transfused red cells with increased rapidity. In those experiments sensitization of the donor dog's cells by the recipient dog's serum eventually was demonstrable, although no incompatibility was detected in vitro by the use of antiglobulin serum following the first three transfusions.

Although sensitization of red cells in vivo may often involve proteins other than gamma globulin,[28, 29] it appears unlikely that any normal serum protein coated the surface of the donor's red cells, since antihuman-serum rabbit serum was employed in the indirect Coombs tests with negative results. The possibility that an abnormal "coating" protein existed in the patients' sera, which was not detected by such standard antiglobulin sera, was investigated by immunizing a rabbit to the serum of Patient 1. However, Coombs serum produced in this way also failed to agglutinate the cells of

Donor 4 after his cells had been incubated with the patient's serum. If circulating or red cell "coating" antibodies existed, the failure to detect them before or after any of the transfusions suggests either that such antibodies were produced only transiently in small amounts and were at once removed on the surface of sequestered red cells, or that the antibodies were fixed to tissues such as the liver and spleen, the sites of red cell sequestration according to body surface radioactivity measurements.

The configuration of the survival curves in each case merits comment. It is widely agreed that red cell survival curves approaching exponential curves, such as those in Case 1, reflect a continuous random destruction of red cells. It is axiomatic that such a process is not one acting diffusely upon all of the red cells but one which is ultimately limited, spatially or stoichiometrically, to a certain fraction of available red cells. Thus, the cells transfused into the first patient appear to have been removed by a limited random process, though admittedly an underlying diffuse injury to the cells may have caused their subsequent fractional removal. An unlimited, diffuse destructive process on the other hand should destroy a homogenous population of red cells in such a way that the incidence of destruction follows a "normal distribution pattern." The summation curve of red cell destruction (and thus of red cell survival) in this instance would be sigmoidal. When the cell population consists of a mixture of cell ages, such sigmoidal curves would become more vertical. Another mechanism creating a sigmoidal pattern of red cell destruction would arise if a lag phase were required for the production of an injurious substance. Either mechanism may be invoked to explain the recurring sigmoidal curves of transfused cells in Case 2. In view of these alternative possibilities, one cannot implicate with certainty either a circulating or tissue-fixed agent of red cell destruction in either patient on the basis of the configurations of the described red cell survival curves.

The significance of the body distribution of the Cr^{51} in these patients, as determined by body surface counts, rests upon studies reported elsewhere[12] which indicate that Cr^{51} accumulates in the spleen to a comparatively great extent only when it is derived from intact Cr^{51}-labeled red cells and not from Cr^{51}-labeled hemoglobin or from circulating plasma protein-bound chromium. The data presented in Figs. 3 and 4 thus indicate that coincident with the disappearance of the transfused red cells from the circulation, there was extensive sequestration of the red cells by the spleen and to a lesser degree by the liver.

The delay in the appearance of an increase in urobilinogen excretion, following removal of the red cells from the circulation of Case 2, is remarkable. Intravenously injected hemoglobin[30] and hematin[31] have been reported to lead to increased fecal urobilinogen excretion within two or three days of their infusion. Similarly, in a normal subject, not reported here, we found the maximum increase in fecal urobilinogen excretion to occur during the first two days after the injection of hemoglobin solution. Furthermore, recent observations employing reticulocytes labeled in vitro with Fe^{59} indicate that

the destruction of sensitized and of agglutinated red cells sequestered by the spleen or by the liver, respectively, begins within several hours of their sequestration.[32] Nevertheless, an eight-day delay separated the mid-point of removal of the transfused red cells from the circulation of Case 2 from the time of the maximum increase in excreted urobilinogen. It is also of interest that 7.65 Gm. of urobilinogen in excess of the base line value was excreted. This exceeds by 4.52 Gm. the amount calculated to have appeared if all the injected heme had been converted to urobilinogen and had been excreted as such. An accelerated destruction of the patient's own red cells may have occurred during the destruction of the transfused cells, although there was no significant change in the daily hematocrit values during this period. In Case 1 no significant rise in urobilinogen excretion occurred during the eleven days following transfusion in which observations were made. Since little is known of the span of time between the disappearance of normal red cells from the circulation and the appearance of derived urobilinogen in the stool, it cannot be stated with certainty whether the delay reported here in the appearance of bile pigment was a physiologic or pathologic consequence. In either event, however, the selectively removed red cells were sequestered in the patient's spleen. Allowing two days for fecal excretion of enterally produced urobilinogen, the indication is that actual destruction of the red cells and release of the derived pigment into the circulation may have been delayed for six or more days after their sequestration in spleen and liver.

That occult red cell incompatibility, demonstrable only in vivo, exists is important to the interpretation of red cell survival studies. It is reasonable to suppose that there may be various degrees of such incompatibility which may mimic clinical extracorpuscular hemolytic processes of various intensities. The interpretation of unexpectedly rapid red cell destruction detected by the Ashby technique in patients who have received many previous transfusions, is presumably most open to question. Under this and other circumstances, the combined use of Cr^{51}-labeled autogenous red cells and of biologically labeled homologous red cells may be necessary for clarification, since each method compensates for the defects inherent in the other.

SUMMARY

Observations are reported on two multiparous women with splenomegaly in whom normal, serologically compatible transfused red cells were found to be incompatible in terms of their survival in vivo. The increasingly rapid removal of homologous red cells from the circulation after successive transfusions and the relatively normal survival of autogenous red cells and of the red cells of one donor suggested the existence of an immune process demonstrable only in vivo. Studies of body Cr^{51} distribution in these patients, following transfusion with Cr^{51}-labeled homologous red cells, revealed that sequestration in the spleen, and, to a lesser extent, the liver, attended differential removal of these cells. The pattern of urobilinogen excretion, however, suggests that actual red cell destruction was delayed for six or more days after such sequestration.

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"NORMAL" SERUM ELECTROLYTES WITH A NOTE ON SEASONAL AND MENSTRUAL VARIATION

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THE use of clinical laboratory examinations in the diagnosis and treatment of disease demands more than a knowledge of their mean normal results. A concept of the normal variation, its degree, its limits, and some of the factors influencing it, and a knowledge of the precision and accuracy of the examinations themselves are required. Technical factors in the examinations explain only a fraction of the variation encountered. Much of it is due to physiologic changes within individuals, and to differences between individuals, groups of individuals, and the populations from which these groups are selected. Such factors as age,[1a] sex,[2, 3] and race[4]; diurnal, menstrual, and seasonal[1b, 5] rhythms; geography, climate, and cultural and dietary habits; and even emotional state[6] might influence the normal variation. Most of these factors are ignored, or assumed to be negligible, in published reports of normal values. While the mean concentrations of the serum electrolytes in "normal" human subjects have been firmly established by a wealth of data accumulated through the years, the normal variation in these values is less well defined, less often discussed, and sometimes overlooked in clinical evaluations. It is, therefore, useful from time to time to restudy samples of an apparently normal population and to relate the results of the chemical findings to as many of the sources of variation as possible. The results of such a study are presented here.

MATERIALS AND METHODS

This general population sample includes 111 apparently healthy volunteers from among the employees of, and visitors to, a single large general hospital in Kansas City, Missouri. Persons of both sexes, ranging in age from 18 to 68 years, contributed blood samples. Only nine Negroes were included; the remainder were Caucasian. Samples were collected from October, 1954, to June, 1955. A study of the annual and menstrual variation was carried out from October, 1954, through September, 1955, on a small group of special volunteers whose numbers and enthusiasm for the study waned as the year waxed long. In all cases blood samples were collected without regard to the time of day or to the state of alimentation. The blood was allowed to clot and, as soon thereafter as possible, it was centrifuged and the serum was separated from the cells. The following analytic methods were used:

1. For sodium and potassium, flame photometry.[7]
2. For calcium and magnesium, an ethylenediaminetetraacetate titration.[8]
3. For bicarbonate, the volumetric procedure of Van Slyke.[9a]
4. For chlorides, a silver nitrate-titrimetric method.[1c]

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5. For phosphate, the photometric method of Fiske and Subbarow.[9b]
6. For protein, a biuret photometric method.[10]

The accuracy of these methods in this laboratory is indicated by the standard deviations of the analyses of multiple replicates of a single large pool of human serum, aliquots of which were run with routine analyses from time to time during the study. In milliequivalents per liter of serum, the standard deviations from the mean values for this serum are: sodium ±2; potassium ±0.1; calcium ±0.09; magnesium ±0.08; bicarbonate ±0.9; chloride ±1.9; inorganic phosphate ±0.1; protein ±0.6. The statistical methods used in the study include the "t" test for the significance of the differences between means,[11] and the analysis of variance and F ratio test for the significance of the variation[12] in the seasonal and menstrual studies.

RESULTS

The serum electrolyte concentrations found in the general population sample are given in Table I. The individual values were distributed in a "normal" manner about the means. When the data were examined for any differences related to the sex of the subjects, statistically significant differences between the mean values for males and those for females were found only for sodium, calcium, and bicarbonate. These data are presented in Table II. The data from the present study are compared with those from six other sources in Table III, and are consistent with the findings of others.

TABLE I. CURRENT SERIES OF "NORMAL" SUBJECTS* SERUM ELECTROLYTE CONCENTRATIONS IN MEQ./L.

ELECTROLYTE	MEAN VALUE	STANDARD DEVIATION	RANGE	
			LOW	HIGH
Sodium	143.0	2.0	128.0	148.0
Potassium	4.4	0.3	3.6	5.3
Calcium	4.8	0.3	4.2	5.5
Magnesium	1.6	0.2	1.1	2.9
Bicarbonate	26.1	1.7	22.6	30.8
Chloride	103.0	3.0	95.0	108.0
Phosphate (inorganic)	2.2	0.3	1.3	3.0
Protein	17.2	1.3	14.6	20.5

*This series consisted of 111 subjects.

TABLE II. ANALYSIS OF SEX DIFFERENCES IN SERUM ELECTROLYTE CONCENTRATIONS (MEQ./L.)

	SODIUM	CALCIUM	BICARBONATE
Males			
Number of subjects	62	76	62
Mean	143.0	4.9	26.4
Standard deviation	3.0	0.3	1.7
Females			
Number of subjects	49	35	49
Mean	144.0	4.7	25.0
Standard deviation	2.0	0.2	1.8
t ratio	2.10*	4.16†	4.15†

*Significant at the 5 per cent level of probability.
†Significant at the 1 per cent level of probability.

In order to study the possible effect of seasonal and menstrual factors, the special volunteers were examined repeatedly at intervals throughout a single calendar year: the male subjects, once each calendar month; the females, at the onset of each menstrual period and on the fourteenth day thereafter. Since the menstrual cycles were of differing lengths and out of

TABLE III. COMPARISON OF DATA FROM SEVERAL SOURCES; RANGE OF ELECTROLYTE CONCENTRATIONS IN MEQ./L. IN NORMAL HUMAN SERUM

	SODIUM		POTASSIUM		CALCIUM		MAGNESIUM		BICARBONATE		CHLORIDE		PHOSPHATE*		PROTEIN†	
	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH
1	128	154	2.9	7.6	4.0	5.8	0.8	2.5	25	30	99	108	1.7	4.9	11.2	19.2
2	132	144	3.6	4.8	4.0	5.5	1.4	2.4	24	31	97	108	2.2	4.4		
3	137	147	4.1	5.6	4.5	5.5	1.6	2.5	22	26	97	106	1.7	2.2		
4	135	148	4.1	5.6	4.5	5.5			24	31	98	108	1.7	2.5		
5	137	148	3.9	5.0	4.8	5.4			25	29	101	106	1.6	2.2		
6	136	145	3.5	5.0	4.2	5.1	1.5	2.5	26	28	100	106	1.7	2.5		
7	128	148	3.6	5.3	4.2	5.5	1.1	2.9	23	31	95	108	1.3	3.0	14.6	20.5

*Assuming a valence of 1.8 for inorganic phosphate: mg. per cent × 0.585 = meq./L.

†Using the Van Slyke factor for protein: Gm. per cent × 2.43 = meq./L.

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phase, the grouping of the female data into months was somewhat arbitrary and artificial. It was done by including in that calendar month all samples obtained within half a month on either side of the date of sampling the male population.

The results for the male group, which initially contained 13 subjects, are presented in Table IV. The variation between the months is significantly greater than the variation between the individual subjects within the months for all the electrolytes except potassium and chloride. The mean value for each electrolyte for each month has been graphed in Fig. 1. No consistent pattern of annual variation is seen and the causes of the variation found are not suggested.

TABLE IV. ANALYSIS OF SEASONAL VARIANCE IN MALES; SERUM ELECTROLYTE CONCENTRATIONS IN MEQ./L.

ELECTROLYTE	MEANS FOR THE GROUP			F RATIO SEASONAL VARIATION	RANGE OF INDIVIDUAL VARIATION			
					GREATEST		LEAST	
	ANNUAL	HIGHEST MONTH	LOWEST MONTH		HIGH	LOW	HIGH	LOW
Sodium	144	147	143	5.08*	154	141	146	142
Potassium	4.3	4.4	4.2	0.67	4.8	3.8	4.3	4.0
Calcium	4.8	5.0	4.6	6.14*	5.6	4.3	5.1	4.6
Magnesium	1.7	1.9	1.5	11.00*	2.4	1.7	1.8	1.5
Bicarbonate	26.7	29.7	25.0	8.29*	30.2	20.4	28.9	26.4
Chloride	103.4	105.7	101.2	0.05	112.5	102.5	105.0	100.0
Phosphate	2.2	2.4	1.8	4.45*	2.9	1.7	2.2	1.8
Protein†	17.5	18.4	16.1	8.19*	18.9	14.6	18.1	16.5

*Significant at the 1 per cent level of probability.

†Using the Van Slyke factor for protein: Gm. per cent × 2.43 = meq./L.

An analysis of the data from the female subjects is shown in Table V. Twelve subjects began this study but only five finished out the entire year. Clustering the data about the dates of examination of the male subjects as

TABLE V. SEASONAL AND MENSTRUAL VARIATION IN FEMALES; SERUM ELECTROLYTE CONCENTRATIONS IN MEQ./L.

ELECTROLYTE	MEANS FOR THE GROUP			F RATIOS		RANGE OF INDIVIDUAL VARIATION			
						GREATEST		LEAST	
	ANNUAL	HIGHEST MONTH	LOWEST MONTH	MENSTRUAL VARIATION	SEASONAL VARIATION	HIGH	LOW	HIGH	LOW
Sodium									
Menses	143	145	141	2.81	8.76†	154	143	145	142
Intermenses	144	146	142			149	140	142	141
Potassium									
Menses	4.2	4.4	4.0	1.00	1.00	4.9	3.9	4.3	3.9
Intermenses	4.2	4.3	4.0			4.6	3.8	4.3	4.1
Calcium									
Menses	4.7	4.9	4.5	1.00	14.60†	5.2	4.4	4.8	4.4
Intermenses	4.7	4.9	4.4			5.0	4.2	4.8	4.6
Magnesium									
Menses	1.7	1.9	1.5	11.00†	107.00†	1.9	1.3	1.9	1.6
Intermenses	1.7	1.8	1.4			2.1	1.4	1.8	1.6
Bicarbonate									
Menses	25.8	27.1	24.7	1.00	11.52†	33.6	24.7	26.0	24.2
Intermenses	25.8	27.6	24.0			33.1	24.2	26.0	23.4
Chloride									
Menses	104.0	106.6	102.6	2.78	5.77†	108.0	96.5	106.0	101.5
Intermenses	103.0	105.0	101.3			110.0	102.5	104.5	101.5
Phosphate									
Menses	2.2	2.4	1.9	5.00*	9.00†	2.8	1.7	2.6	2.3
Intermenses	2.3	2.6	2.0			3.2	1.8	2.3	1.8
Protein‡									
Menses	16.7	17.9	16.0	0.90	6.70†	18.9	14.9	18.1	16.5
Intermenses	16.7	18.0	15.3			19.1	15.4	18.1	16.0

*Significant at the 5 per cent level of probability.
†Significant at the 1 per cent level of probability.
‡Using the Van Slyke factor for protein: Gm. per cent × 2.43 = meq./L.

medians resulted in 11 monthly samples rather than 12. This was the first source of variation in the analysis. The mean value of all samples collected at a menstrual period and the mean value of all intermenstrual samples within each "month" was a second source of variation. The differing number of subjects in each of the categories thus established made the inclusion of the individual variation as a third source in the analysis of variance prohibitively

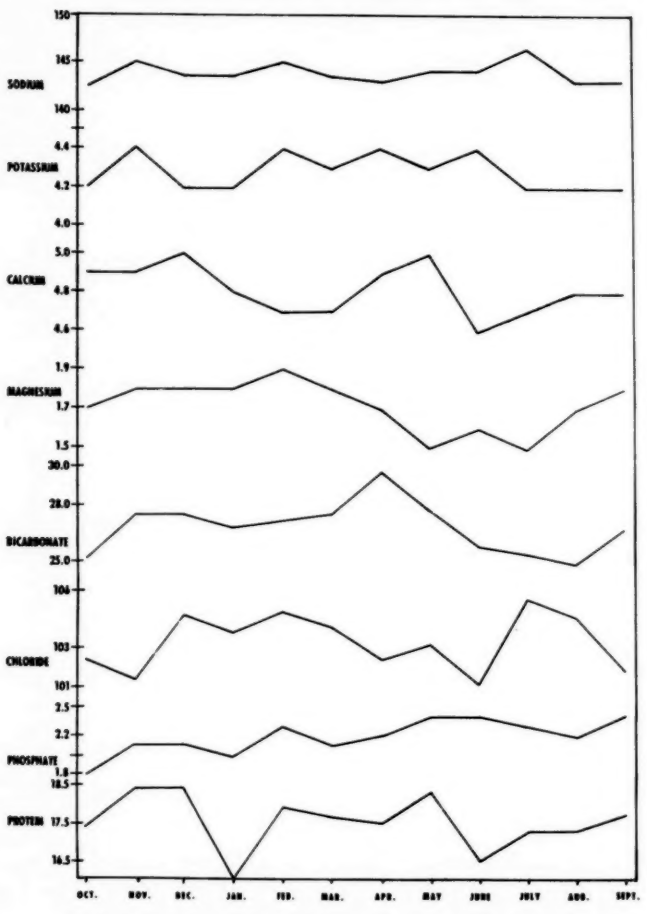

Fig. 1.—Serum electrolytes: annual variation in men.

complex, and its effect on the results, therefore, could not be fully determined. The patterns of variation throughout the year for the female group are shown in Fig. 2 where the mean values for the menstrual and for the intermenstrual samples are plotted against the dates of collection. Calcium, magnesium, and chloride are seen to follow a sine-like pattern over the year. Clear-cut patterns of annual variation for the other electrolytes are not so readily apparent; yet the variation was significant for all except potassium. In the case of magnesium and of phosphate there was a significant variation between the menstrual and intermenstrual bloods as well, magnesium tending to be higher

and phosphate lower at the menses than at the intermenses. These differences were, however, not consistent and appeared to interact with the seasonal fluctuations.

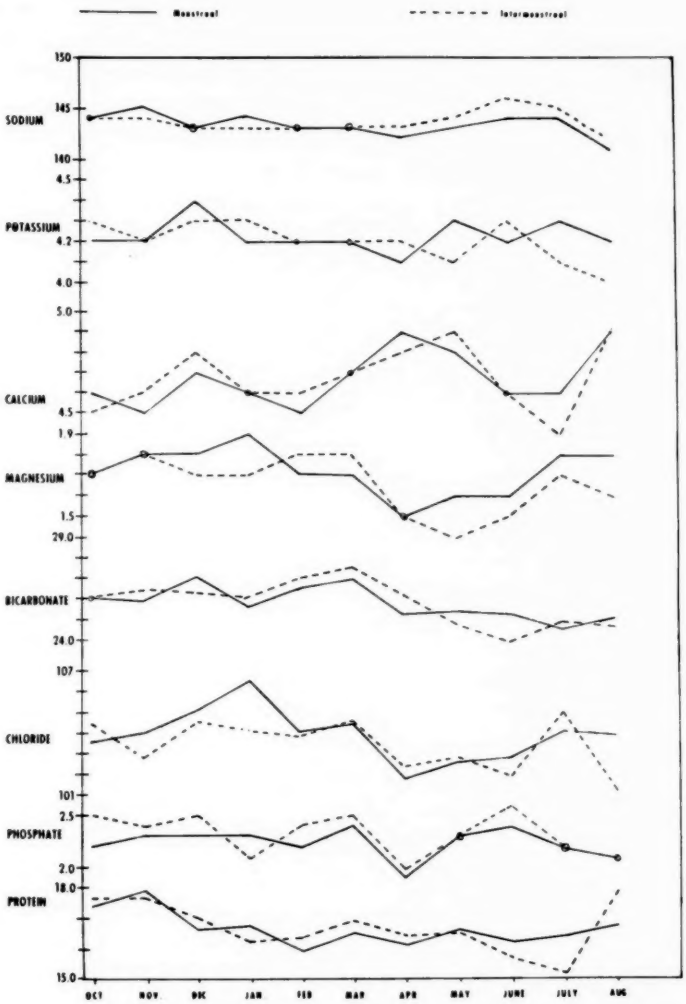

Fig. 2.—Serum electrolytes: annual and menstrual variation in women.

DISCUSSION

It is evident from these findings that seasonal and menstrual rhythms produce some of the variation normally encountered in the serum electrolyte levels of any individual. However, the group of subjects studied is too small, and a single annual cycle is too short to allow speculation as to the mechanisms responsible for the variation demonstrated. It would be of interest to carry such a study as this through several consecutive years, but with human volunteers such an undertaking is formidable. It is hoped that observations made in the future will further elucidate the influence of seasonal and menstrual rhythms on serum electrolyte concentrations.

SUMMARY

An analysis of the serum electrolyte concentrations of a group of apparently healthy human volunteers is presented. Blood for analysis was drawn without regard to the time of day or to the state of alimentation. These data are consistent with the normal values reported by others. Sex differences in the concentrations of sodium, calcium, and bicarbonate are demonstrated. In a small group of male volunteers followed for a single year, significant annual variation in the serum concentrations occurred for all electrolytes except potassium and chloride; however no consistent pattern of variation was discernible. In a small group of female volunteers similarly studied, significant annual variation was demonstrated for all electrolytes except potassium. A sine-like pattern of change was seen for calcium, magnesium, and chloride. Significant variation also was found between the serum concentrations at the menses and at the intermenstrual period for magnesium and for phosphate. The variation for these two ions with menstrual cycles appears to be interactive with that related to the annual cycle. These data help to define the normal variation encountered in serum electrolyte concentrations.

We wish to express our appreciation for the able technical assistance of Miss Betty Huffman, Miss Mary Lou Brosnahan, and Mrs. Marjorie Wight, and for the assistance of Mrs. Peggy Brunkhorst in the preparation of the manuscript. We are especially indebted to Dr. John H. McCormack for his assistance with the statistical analyses.

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THE RENAL TUBULAR REABSORPTION AND URINARY EXCRETION OF CALCIUM BY THE DOG

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STUDIES of renal mechanisms involved in the tubular reabsorption and excretion of calcium are complicated by the fact that this ion species is in part bound to the plasma proteins. In order to calculate the quantity of calcium which enters the glomerular filtrate per unit of time, one must measure not only the rate of formation of filtrate and the plasma calcium concentration but also the fraction of calcium which is freely diffusible, i.e., filterable. Jahan and Pitts[1] used high pressure ultrafiltration through cellophane to determine this moiety. Since no precautions were taken to prevent loss of carbon dioxide, the diffusible fraction may have been somewhat underestimated.

They maintained that in the dog over 99 per cent of the calcium normally filtered is reabsorbed. Furthermore, when the plasma calcium concentration and the urinary excretion of calcium were both increased by the administration of parathyroid hormone, tubular reabsorption was also increased. More recently Chen and Neuman,[2] utilizing a centrifuge type of ultrafilter with which pH could be controlled, also observed that the reabsorption of filtered calcium is more than 99 per cent complete under normal circumstances. Furthermore, rate of reabsorption varied directly with rate of filtration of calcium and no tubular maximum for reabsorption was observed.

In the experiments described below, excretion and reabsorption of calcium were studied in the dog over a range of plasma concentration from 8 to 37 mg. per cent. The diffusible fraction was estimated graphically from the measured total plasma calcium concentration, pH, and protein content according to relationships previously established by a system of compensated dialysis.[3] Although urinary excretion increases as plasma calcium is elevated, reabsorption likewise increases. No reabsorptive maximum was observed.

METHODS

The seven experiments forming the basis of this report were performed on seven mongrel female dogs, lightly anesthetized with intravenous sodium pentobarbital. Five preliminary experiments, although yielding results qualitatively in agreement with those considered here, were in one respect or another incomplete. Infusions containing creatinine, dextrose, and varying amounts of calcium chloride or gluconate were administered intravenously at a constant rate of 5 ml. per minute. The following values were found useful

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as a rough guide to the infusion of calcium. To raise the plasma calcium concentration by 1 mg. per cent, one must administer a priming dose of 2 mg. of calcium per kilogram and a sustaining infusion of 0.02 mg. of calcium per kilogram per minute.

After an equilibration period of 30 minutes following the prime and the start of an infusion, two 15-minute urine collections were made. A new prime and infusion were then given to raise the plasma calcium concentration in stepwise fashion. Standard clearance procedures, including arterial blood sampling and urine collection by catheter with bladder washout, were used. Chemical methods employed have, for the most part, been described in previous communications.[4, 5] However calcium was determined in plasma and urine by an indirect flame photometric method,[6] and the filterable fraction of calcium was interpolated from a graph relating this moiety to plasma calcium, pH, and protein as determined by a system of compensated dialysis.[3]

RESULTS

The infusion of either calcium chloride or calcium gluconate in quantities sufficient to elevate the plasma calcium concentration significantly was frequently, though not invariably, accompanied by a precipitous fall in filtration rate and by the development of oliguria. Uniformly, acidosis, hyponatremia, and hypokalemia developed. Because it was felt that circulatory disturbances arising in the cardiac irregularities induced by hypercalcemia might be accentuated by the fall in plasma potassium, potassium chloride was given in later experiments. Unfortunately, prevention of hypopotassemia did not prevent the fall in filtration rate.

In Table I are presented the significant data from two experiments relative to calcium excretion and reabsorption. In experiment 8, little change in filtration rate was observed; in experiment 11, a marked fall occurred. In both, enough potassium chloride was given to increase the plasma potassium above its initial level.

TABLE I. RELATIONSHIP BETWEEN PLASMA CONCENTRATION AND EXCRETION AND REABSORPTION OF CALCIUM IN TWO DOGS INFUSED WITH CALCIUM CHLORIDE

	URINE FLOW (ML./MIN.)	GLOM. FILTRATE (ML./MIN.)	CALCIUM					
			PLASMA CONC. (MG. %)	DIFFUSIBLE FRACTION	EXCRETED (MG./MIN.)	REABSORBED (MG./MIN.)	REABSORBED (% OF FILT.)	CLEARANCE (ML./MIN.)
			Exper. 8; Dog Weight 21.7 Kilograms					
1.	1.80	58.7	11.1	0.57	0.03	3.72	99.2	0.27
2.	1.47	54.3	11.0	0.57	0.02	3.41	99.4	0.18
3.	0.44	43.6	21.8	0.68	0.04	6.49	99.4	0.16
4.	0.33	47.9	22.4	0.69	0.05	7.33	99.3	0.23
5.	2.66	58.0	27.9	0.71	0.64	10.8	94.5	2.29
6.	3.94	56.7	28.5	0.71	0.81	10.7	93.0	2.83
7.	6.53	54.8	36.4	0.72	2.30	12.1	84.1	6.32
8.	6.53	48.0	37.0	0.72	2.21	10.6	82.8	5.98
			Exper. 11; Dog Weight, 15.0 Kilograms					
1.	1.26	49.8	11.2	0.70	0.02	3.80	99.4	0.20
2.	1.00	48.7	11.7	0.71	0.02	3.99	99.5	0.18
3.	0.50	28.9	22.5	0.81	0.08	5.18	98.5	0.36
4.	0.43	23.6	22.8	0.81	0.06	4.33	98.6	0.26
5.	0.53	18.0	31.4	0.83	0.09	4.64	98.0	0.31
6.	0.83	19.6	32.8	0.82	0.15	4.75	96.9	0.46

In experiment 8, little change in glomerular filtration rate was observed; in experiment 11, a marked fall occurred.

It is evident from experiment 8 that increasing plasma calcium in stepwise fashion to a level more than 3 times the normal increased calcium excretion from 0.02 to 2.3 mg. per minute. While this increase is very great percentagewise, it is not so impressive in absolute terms, especially in view of the fact that reabsorption increased from 3.4 to 12.1 mg. per minute. However, reabsorption, as per cent of filtered calcium, decreased from 99 to 83 per cent. In this and in one other experiment in which filtration rate remained relatively constant, no evidence of saturation of a tubular reabsorptive mechanism was evident over a wide range of plasma concentration.

Experiment 11 illustrates the changes in reabsorption and excretion of calcium as a consequence of tripling plasma concentration in an experiment complicated by a marked fall in glomerular filtration rate. Again, though excretion increased some sevenfold, the absolute change was not impressive. Reabsorption increased as a consequence of elevation of plasma calcium, but not to the extent seen in experiment 8, because the fall in filtration rate diminished the quantity of calcium filtered. Of the seven experiments forming the basis of this report, five were complicated by falls in filtration rate.

The results obtained in seven experiments are summarized in Fig. 1 in which calcium reabsorption as per cent of calcium filtered is plotted as a function of plasma calcium concentration. A decrease in per cent reabsorption is evident as plasma concentration is raised.

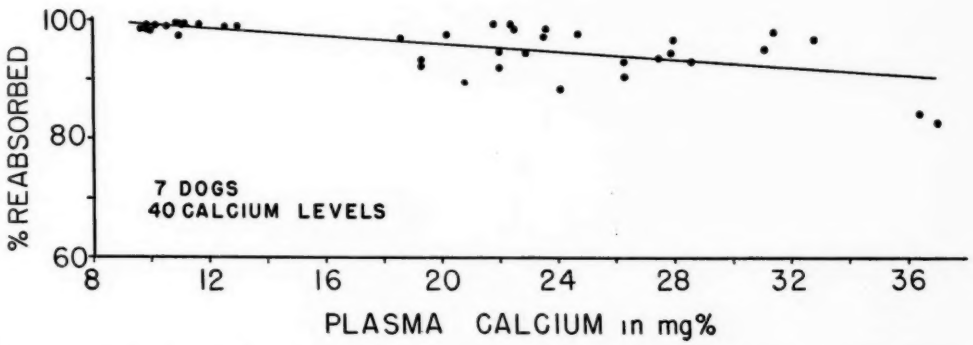

Fig. 1.—Relation between per cent of reabsorbed filtered calcium and plasma calcium concentration.

In Table II are summarized the changes in plasma composition observed in experiments 8 and 11 as a consequence of infusion of calcium solutions at a rate of 5 ml. per minute. A marked acidosis developed in these and in the other experiments as well. This acidosis is only in part metabolic in character, for the decrease in plasma bicarbonate, at least in experiment 8, is relatively minor. In fact a respiratory component is evident in the acidosis of both experiments, but whether due to depression of the respiratory center or to depression of circulatory or lung function is unknown. Roughly, hyponatremia developed in proportion to hypercalcemia. Chloride did not change in any characteristic fashion, nor did the sums of the anions relative to the sums of the cations reveal the accumulation of any undetermined ions.

DISCUSSION

It is evident from our experiments as well as from those previously published[1, 2] that the reabsorption of calcium from the glomerular filtrate under normal conditions is more than 99 per cent complete. Elevation of plasma calcium results in increased calcium excretion, but the increase in the quantity excreted is much less than the increase in the quantity filtered. Accordingly, reabsorption rises and no tubular maximum for reabsorption can be demonstrated.

A more detailed analysis of the reabsorptive mechanism is difficult, for the infusion of calcium commonly causes a fall in glomerular filtration rate. Such a fall has been described, although not documented, by Chen and Neuman.[2] It probably underlies the "negative diuresis" observed by Adolph.[7] The fall in glomerular filtration rate may be related to myocardial insufficiency, cardiac arrhythmia, and fall in blood pressure. We were unable to antagonize the effects of calcium by the administration of potassium. Associated with the infusion of calcium are acidosis, hyponatremia, and in some instances a moderate increase in plasma phosphate. It is probable that these abnormalities in composition of the body fluids contribute to the circulatory insufficiency and to the fall in filtration rate.

TABLE II. CHANGES IN PLASMA COMPOSITION DURING THE INFUSION OF CALCIUM CHLORIDE IN THE SAME TWO EXPERIMENTS SUMMARIZED IN TABLE I

	pH	SODIUM	CALCIUM	POTASSIUM	CHLORIDE	BI-CARBONATE	PHOSPHATE	PROTEIN	SUM CATIONS	SUM ANIONS
		MEQ./LITER					mM/L	GM. %	MEQ./LITER	
					Experiment 8					
2.	7.40	136	5.50	3.17	107	18.4	0.83	6.85	145	143
4.	7.28	130	11.2	3.42	105	16.2	1.61	6.40	144	139
6.	7.19	126	14.3	4.03	100	15.8	1.79	6.36	144	134
8.	7.12	114	18.5	4.22	104	15.3	1.68	6.40	137	137
					Experiment 11					
2.	7.38	144	5.84	3.64	113	16.3	1.42	5.55	153	145
4.	7.20	128	11.4	6.04	113	11.4	1.64	4.36	145	137
6.	7.02	126	16.4	5.73	116	10.8	1.57	2.87	138	137

SUMMARY

Renal tubular reabsorption and urinary excretion of calcium have been studied in the dog over a range of plasma concentration from 8.3 to 37 mg. per cent. Normally, more than 99 per cent of the filtered calcium is reabsorbed by the renal tubules. In a majority of experiments, the infusion of calcium chloride or gluconate in the amounts necessary to attain high plasma concentrations caused a sharp fall in glomerular filtration rate and led to oliguria. However, in two experiments it was possible to triple plasma calcium with minimal change in filtration rate. In these experiments tubular reabsorption increased nearly in proportion to the increased rate of delivery of calcium into the glomerular filtrate. Accordingly, no tubular reabsorptive maximum was observed. In all experiments, marked acidosis and hyponatremia developed.

I wish to express my appreciation to Dr. Robert F. Pitts for assistance in performing this work and in preparing the manuscript.

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STUDIES ON HUMAN SERUM GAMMA GLOBULIN

I. Half-Life and Rate of Production

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ONE of the results of the studies on Rh sensitization has been the development of a serologic method for determining gamma globulin concentration in serum and other fluids.[1] The principle of this method is to measure the ability of the serum to inhibit the agglutination by rabbit immune anti-human globulin serum of human **Rh$_0$**-positive red blood cells coated with **Rh$_0$** antibody. The highest saline dilution of the unknown serum capable of inhibiting the agglutination of the coated red cells of such a system (inhibition titer) is determined in parallel with that of a standard normal human serum. The ratio of the inhibition titer of the unknown serum to that of the standard multiplied by one hundred gives the gamma globulin concentration of the unknown serum expressed as a percentage of the normal. In a previous paper,[2] it was shown how this method could be applied as a diagnostic test for agammaglobulinemia, and to follow the results of treatment of the patient by injection of human gamma globulin. The purpose of the present paper is to describe further studies on gamma globulin in human serum with special reference to the half-life of gamma globulin and the rate of production of gamma globulin by the body.

CASE REPORT

The subject was a 58-year-old Negro woman who weighed 110 pounds. She had had 17 previous admissions to Bellevue Hospital because of periodic episodes of lobar pneumonia and bronchopneumonia. Forty or more pyrexic episodes had been documented since 1941. Her blood groups were B, N, Rh$_0$, but her serum contained no anti-A agglutinins detectable in tests either in saline or in acacia. Tests on her saliva showed her to be a secretor of group B substance. By the gamma globulin inhibition technique carried out on several occasions, the gamma globulin concentration of her serum was found to range from 2.5 to 5 per cent of normal.

In November, 1955, the patient was readmitted with a low-grade fever, caused by a recurrence of the pneumonic infection. The patient was treated with antibiotics until she became afebrile. After four days without therapy, on December 2, 1955, the experimental blood transfusion was carried out. For this purpose, blood of the groups A_2, M, k, Rh$_1$rh, preserved in acid-citrate-dextrose and stored at 4° C. for 4 days, was used. Group A blood was chosen deliberately in order to test the prediction that such blood would survive normally in the patient's circulation, even though she belonged to group B. To prevent any untoward reaction, we added Benadryl to the blood, and at the onset of the transfusion we gave the patient acetylsalicylic acid. Since the patient was not anemic, in order that her circulation might not be overloaded, 500 ml. of her blood was withdrawn

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at the same time the 500 ml. of transfused blood was given. The patient withstood the transfusion well except for one short period during which she complained of mild chilliness, although there was no actual chill or rise in temperature.

Blood serum was obtained before and after the blood transfusion and at intervals of several days or longer for periods up to two months for determination of gamma globulin by the inhibition technique.[1] Since gamma globulin inhibition titrations, in common with other serologic titrations, are subject to a large experimental error, each determination was carried out several times and the results were averaged. Moreover, four of the serum specimens were tested on two separate occasions, while one specimen was tested on three separate occasions.

RESULTS

The icterus index before the transfusion was 2 units, and after the transfusion 4 units, thus showing no significant rise. Differential agglutination tests showed prolonged survival of the donor's red blood cells, since the transfused cells could still be demonstrated in the patient's circulation for as long as 60 days after the transfusion. At no time could any anti-A agglutinins be demonstrated in the patient's serum.

On the day following the transfusion, the patient's temperature rose abruptly to 105.6° F. This rise was attributed to an antigen-antibody reaction between "natural" antibacterial antibodies passively acquired from the donor and the bacteria in the patient's body. When antibiotics were administered, the patient's temperature gradually returned to normal.

The results of gamma globulin inhibition titrations are summarized in Table I. Table II is an illustrative protocol of inhibition titrations carried out in parallel on a normal adult serum, the patient's serum obtained on Dec. 2, 1955, immediately before the transfusion, the patient's serum obtained the day following the transfusion, and serum obtained from the patient on Jan. 23, 1956, which was almost two months after the blood transfusion. Table II shows clearly the different inhibition titers obtained with these four different specimens of serum.

TABLE I. APPROXIMATE CONCENTRATION OF GAMMA GLOBULIN IN THE PATIENT'S SERUM BEFORE AND AFTER TRANSFUSION

SPECIMEN OF	DATE SPECIMEN OBTAINED	GAMMA GLOBULIN CONCENTRATION PER CENT OF NORMAL			AVERAGE
		1	2	3	
Donor	12/ 2/55	100	100	100	100
Patient (pretransfusion)	12/ 2/55	2.4			2.4
Patient (midtransfusion)	12/ 2/55	15.4	15		15.2
Patient (end of transfusion)	12/ 2/55	20			20
Patient (posttransfusion)	12/ 3/55	11	12	13	12
	12/ 5/55	11	9		10
	12/ 7/55	12			12
	12/12/55	7.5			7.5
	12/27/55	11.4	9		10.2
	1/ 4/56	11			11
	1/11/56	8.6			8.6
	1/23/56	6	6.1		6

The concentration of gamma globulin in the donor's serum was arbitrarily set equal to 100 per cent. Each determination given in the table represents the average of at least two titrations. The values given in columns 1, 2, and 3 represent determinations on the same sample of serum carried out on different days.

TABLE II. RESULTS OF INHIBITION TITRATIONS ON PATIENT'S SERUM, SHOWING SURVIVAL OF GAMMA GLOBULIN RECEIVED BY TRANSFUSION

SPECIMEN OF SERUM TESTED	REACTION OF ANTI-GLOBULIN SERUM WITH Rh_0-POSITIVE CELLS COATED WITH Rh_0 ANTIBODIES, AFTER MITXURE WITH SPECIMEN IN DILUTION									
	UNDIL.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Normal adult	–	–	–	–	–	–	–	–	tr.	±
Patient (pretransfusion, Dec. 2, 1955)*	–	–	tr.	±	++	++	++	++	++	++
Patient (day after transfusion, Dec. 3, 1955)*	–	–	–	–	–	tr.	++	++	++	++
Patient (specimen obtained Jan. 23, 1956)	–	–	–	tr.	+	++	++	++	++	++

*This specimen had been saved in a freezer so that it could be titrated coincidently with the January twenty-third specimen.

DISCUSSION

Since an individual's blood volume is approximately one-thirteenth to one-fifteenth of the body weight, the blood volume of the subject was approximately 3,500 ml. Thus, the plasma from the 500 ml. of blood which the patient received by transfusion underwent approximately a sevenfold dilution. Considering that the patient originally had a gamma globulin concentration of approximately 2.5 per cent of normal, one would expect that after the transfusion the gamma globulin concentration would be increased by 14.3 per cent to about 17 per cent of normal. The gamma globulin concentration immediately after the transfusion was found to be approximately 20 per cent of normal. Considering the limits of error of the method and calculations, this is a relatively satisfactory agreement. After the transfusion, gamma globulin would be expected to diffuse into the tissue spaces until the gamma globulin concentration inside and outside the blood vessels is equalized. As shown in Table I, the gamma globulin concentration stabilized at about 11 per cent of normal. If from this concentration the patient's original gamma globulin concentration were subtracted the indication would be that the gamma globulin received by transfusion underwent a total dilution in the patient's body of approximately 12 times. Assuming that gamma globulin diffuses freely from the blood vessels, this observation indicates that the volume of tissue fluid into which the gamma globulin diffuses is somewhat less than the volume of the plasma within the blood vessels.

If one assumes that all gamma globulin, like **Rh_0** antibodies,[3] has a half-life of 30 to 35 days, one would expect the gamma globulin concentration to be halved after a month. However, considering that the subject was producing enough gamma globulin of her own to yield a steady concentration of about 2.5 per cent of normal, the expected concentration after one month would be half of 11 per cent plus 2.5 per cent, or about 8 per cent of normal. Similarly, after 2 months the gamma globulin concentration would be expected to drop to 6.5 per cent of normal. As shown in Table I, the results obtained roughly approximate the values expected under the hypothesis of a half-life

of approximately one month for gamma globulin. Similar observations have been reported by Barandum and associates[4] on an agammaglobulinemic patient treated by injections of gamma globulin.

The findings just presented also make it possible to estimate the amount of gamma globulin in the entire body and the amount that must be produced daily for homeostasis. An adult weighing about 150 pounds has a blood volume of approximately 5,000 ml. and a plasma volume of about 2,750 ml. If one makes the assumption, based on the findings just described, that the volumes of plasma inside and outside the blood vessels are about equal, the total volume of plasma in the body becomes equal to approximately 5,500 ml. Assuming the concentration of gamma globulin in the plasma to be 1 Gm. per cent, the total amount of gamma globulin in the body of an adult of average weight would then be equal to about 55 Gm., or approximately four-fifths of a gram per kilogram.

A half-life of gamma globulin of 35 days implies that every day approximately one-fiftieth of the gamma globulin is destroyed. Thus, a little over 1 Gm. of gamma globulin is destroyed each day and must be replaced in the normal individual to maintain homeostasis. This implies that the body produces about 16 mg. of gamma globulin per kilogram every day. In the patient whose gamma globulin concentration was only 2.5 per cent of normal, the rate of production must have been about one-fortieth of normal, or only about 0.4 mg. of gamma globulin per kilogram per day.

Of interest is the fact that even though the subject belonged to group B, the transfused group A blood cells survived in her circulation and did not stimulate the production of anti-A agglutinins. Thus, the gamma globulin received by transfusion did not convey the capacity to become isoimmunized. This observation indicates that antibodies are not produced by a denaturing process or by any other process altering the configuration of already preformed gamma globulin molecules, but rather they result from some change within the antibody-producing cells which affects the synthesis of gamma globulin molecules in a way that gives them the attributes of antibodies. Thus, any beneficial results which follow gamma globulin therapy in agammaglobulinemia must be attributed to the passive transfer of immunity from the donors and must not be attributed to the acquisition of a capacity to form anti bodies.

SUMMARY

The gamma globulin inhibition technique has been applied to the study of the half-life of gamma globulin in a patient with agammaglobulinemia, to whom a blood transfusion was given. The results obtained are compatible with the estimate of a half-life for gamma globulin of approximately 30 to 35 days as determined previously from studies on $\mathbf{Rh_0}$ antibodies. It is calculated that the total amount of gamma globulin in an adult of average weight is about 55 Gm., or about four-fifths of a gram per kilogram. The rate of gamma globulin production necessary to maintain homeostasis is estimated to be approximately 16 mg. per kilogram per day.

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EFFECT OF CORTICOIDS UPON THE RESISTANCE OF THE KIDNEY TO SUBLIMATE INTOXICATION

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EARLIER observations have shown that the syndrome of nephrocalcinosis, induced by the administration of acid sodium phosphate (NaH_2PO_4), in the rat, is aggravated by desoxycorticosterone acetate (DOCA) and inhibited by cortisol acetate (COLA). When both hormones are simultaneously administered, they mutually antagonize each other in this respect.[1-4]

It is noteworthy that the histologic changes in the kidneys of rats receiving dietary excesses of sodium phosphate are very similar to those induced by corrosive sublimate ($HgCl_2$). Furthermore, experiments on mice have shown that a steroid hormone, testosterone, can protect the kidney against the damaging effect of sublimate.[5]

In view of these findings it seemed of interest to determine the possible influence of DOCA and COLA upon the nephrocalcinosis produced by sublimate.

METHODS

Forty female Sprague-Dawley rats, having a mean initial body weight of 106 grams (range: 98-115 grams), were subdivided into 4 equal groups of 10 rats each, and treated with 2 mg. per day of DOCA and/or COLA microcrystals (administered subcutaneously in 0.2 ml. aqueous vehicle), as indicated in Table I, during the entire period of observation. After five days of this treatment, the animals of all groups received 1 mg. of $HgCl_2$ in 0.2 ml. of water subcutaneously on four consecutive days. It will be noted that this dose is well above the LD 50.

Throughout the experiment, the rats were fed exclusively "Purina Fox Chow" and given tap water to drink. The experiment was terminated on the fifteenth day by killing the animals with chloroform. At autopsy, one kidney was fixed in neutral formalin (for the histochemical demonstration of calcium deposit with Kossa's silver nitrate technique), the other in Susa solution (for subsequent staining with the PAS-technique[6]).

TABLE I. EFFECT OF CORTICOIDS UPON CERTAIN MANIFESTATIONS OF SUBLIMATE INTOXICATION

GROUP	HORMONE TREATMENT	GAIN IN BODY-WEIGHT (%)	RENAL CALCIFICATION	CECAL ULCERS	MORTALITY (%)
1	None	+ 22	2.0	1.3	40
2	COLA	− 15	0	2.7	70
3	DOCA	+ 5	2.2	1.1	10
4	COLA + DOLA	− 15	0	1.8	20

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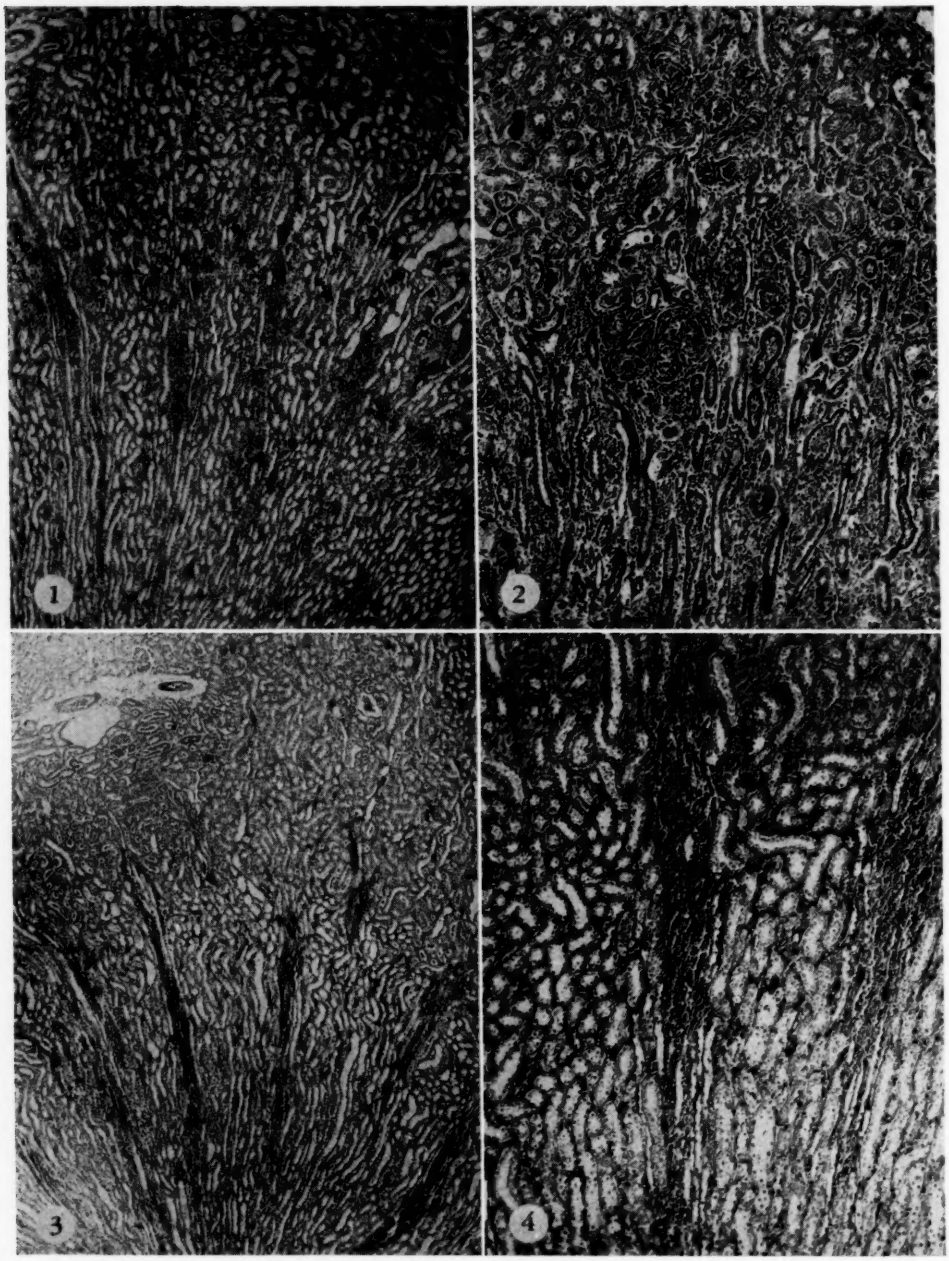

Fig. 1.—Calcium depositions limited to the "zona intermedia" of the kidney in a rat of Group 3 treated with DOCA and sublimate (Kossa's stain; ×50; reduced ⅙).

Fig. 2.—Higher magnification of a region from the kidney illustrated in Fig. 1. to show the intense disorganization of the "zona intermedia," with granuloma formation around degenerating tubules (PAS-stain; ×80; reduced ⅙).

Fig. 3.—Kidney of a rat of Group 4 treated with DOCA and COLA during sublimate intoxication. Complete absence of calcified material (Kossa's stain; ×50; reduced ⅙).

Fig. 4.—Higher magnification of a region from the kidney illustrated in Fig. 3. Note the essentially normal appearance of the tubules in the "zona intermedia" (PAS-stain; ×80; reduced ⅙).

RESULTS

Macroscopic inspection of the cut surface of the *kidney* revealed a chalky white deposit, sharply limited to the region designated by Peter[7] as the "inner stripe of the outer zone of the medulla," and by Heidenhain[8] as "zona intermedia." This stratum consists exclusively of unbranching collecting tubules and loops of Henle. The whitish zone was most conspicuous in the rats receiving no hormone treatment (Group 1), or only DOCA injections (Group 3). It was not distinguishable either in the animals given exclusively COLA (Group 2) or in those receiving both COLA and DOCA (Group 4).

Histologic observation of slides stained with Kossa's silver nitrate technique showed pronounced calcium deposits in the renal tubules within the "zona intermedia," in Groups 1 and 3 (Figs. 1 and 2). It is difficult to identify with certainty the part of a nephron in which massive calcium deposits are located, because the epithelium tends to be damaged and often is virtually destroyed by an intense foreign-body granuloma. Yet, as judged by initial stages of the calcium deposition process, it seems to us that calcified casts are lodged almost exclusively in the thick segments of Henle's loops. Virtually no calcification was observed in the cortex and in the papilla of the kidney in any of our animals. Apart from these structural details concerning the location of the calcium deposits, our histologic study merely confirmed the macroscopic diagnosis. It showed that in the absence of steroid hormone treatment or after treatment with DOCA alone, sublimate causes marked renal calcification, with reactive granuloma formation around the degenerating and calcified parts, while treatment with COLA prevents this change, both in otherwise untreated rats and in animals simultaneously receiving DOCA (Figs. 3 and 4). In Table I, the mean intensity of nephrocalcinosis is listed after assessing the degree of calcification in each rat, in a scale of 0 to 3.

Fig. 5.—Section through the cecum of a rat of Group 2, which was treated with COLA alone. The large light semilunar area in the cecal wall (middle of field), just above the obliquely cut ileum (lower right corner), is necrotic material within the crater of the ulcer (PAS-stain; ×20).

As an incidental observation, we noted *necrotizing ulcers in the cecum,* just below the entrance of the ileum into the large intestine. Such ulcers, sharply localized to this particular region, were found to be a constant accompaniment of sublimate intoxication in our earlier experiments and, in the present series, they were again demonstrable in every animal. Their severity was greatest in four rats which died during the first few days of sublimate treatment with manifestations of severe cachexia. At this time, the entire cecum became intensely edematous and hemorrhagic. These lesions were perhaps largely responsible for much of the initial mortality. We have attempted to estimate the intensity of these exulcerations at autopsy (also in a scale of 0 to 3), in the animals which survived until the end of the experiment. The mean severity of the ulcers, as expressed in this scale, is listed in Table I. It will be noted that, in the groups in which renal calcification was prevented by COLA, the intensity of the cecal ulcers was greatest.

The *mortality* was actually increased in Groups 2 and 4, in which COLA treatment prevented nephrocalcinosis. This may have been due to the fact that, under our experimental conditions, renal damage was not the principal cause of death, or that the aggravation of the cecal lesions by COLA counterbalanced the beneficial action of this steroid upon the kidney.

SUMMARY

The calcification and secondary foreign-body granuloma formation in the kidneys of rats poisoned with corrosive sublimate were prevented by cortisol acetate (COLA), but not by desoxycorticosterone acetate (DOCA).

In rats treated simultaneously with equal amounts of COLA and DOCA, the kidney-protecting effect of the former was not inhibited by the latter compound.

As an incidental observation, it was found that the necrotizing ulcers of the appendix, which develop in rats given large amounts of sublimate, were actually aggravated by COLA and not significantly influenced by DOCA.

Evidently, the renal manifestations of mercurial intoxication can be rather selectively inhibited by a potent glucocorticoid hormone.

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COMPARISON OF THE CHANGE IN THE RED CELL POTASSIUM CONTENT WITH THE TOTAL BODY BALANCE OF POTASSIUM DURING POTASSIUM THERAPY

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POTASSIUM deficiency of cells is usually suspected when there is a history of anorexia, vomiting, or diarrhea and is thought to be present if the extracellular potassium concentration is reduced. However, loss of potassium from cells is not always associated with a reduction in the extracellular potassium concentration, and a reduction in the extracellular potassium concentration can occur without loss of potassium from cells.[1] Therefore, the direct analysis of cells appears to be the best method currently available for rapidly estimating the cell content* of potassium.

Knowledge of the potassium content of muscle cells is particularly important in appraising the total body status of cell potassium content, since muscle tissue contains over 50 per cent of body potassium, and muscle cells have been shown to lose potassium in potassium deficiency. However, muscle biopsy in humans is not feasible for routine clinical use.

Potassium deficiency of red blood cells has been reported in diseases known to be associated with body cell potassium depletion,[2-13] but few data are available indicating the concurrent potassium status of red cells and other body cells in these diseases. In the present study, concurrent measurements of the red cell potassium content and of the total body balance of potassium were made in 14 patients with disturbances of potassium metabolism in order that the relationship of the change in potassium of red cells, per kilogram solids, and of the total body balance of potassium might be seen.

CLINICAL MATERIALS AND METHODS

Patients with suspected potassium deficiency due to gastrointestinal disorders, diabetic acidosis, and nephritis were studied. Complete potassium and nitrogen balance data—including analysis of food and stools—were obtained in three cases (Nos. 4, 5, and 9). In eleven patients stool analyses were not made, and the nitrogen and potassium content of ingested food was estimated from standard tables.[14] In five patients (Nos. 1, 6, 8, 13, and 14) nitrogen balances were not made; this omission would not appear to affect materially the calculated cell balance of potassium since the periods involved were of

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*Hereafter red cell potassium *content* will refer to the millimoles of potassium per kilogram red cell solids, whereas red cell potassium *concentration* will refer to the millimoles of potassium per liter of cells or cell water.

short duration and the body weight remained relatively constant. None of the patients had diarrhea except No. 2 who had one loose stool. Most of the potassium was given intravenously to Patients 2, 6, 8, and 10 because of anorexia. Blood samples were drawn before potassium therapy and again at least four hours after the last administration of potassium.

The chemical methods used to analyze plasma and red cells and the analytic variability obtained by these methods are reported in detail elsewhere.[15] The micro-Kjeldahl method was used for the analysis of nitrogen. Cumulative balance errors may be as much as 15 to 30 mM of potassium per day in the incomplete balances because of unmeasured stool losses and variation in the potassium content of food, but are probably less than 5 mM of potassium per day in the complete balances.

CALCULATIONS USED FOR DERIVING THE CHANGE IN THE POTASSIUM CONTENT OF THE AVERAGE BODY CELL AND OF RED CELLS

The balance of potassium of all body cells was assumed to be the total body balance of potassium corrected for a change in the extracellular potassium content and, when measured, for a change in the nitrogen balance, with use of a K:N ratio of 2.8 mM per gram. It was assumed that the volume of extracellular fluid was 20 per cent of body weight. Since the body weight of most of the patients remained relatively constant and the periods were relatively short, a large change in the extracellular volume would appear to be unlikely in all but the patients with diabetic acidosis, in whom a maximal error in the calculated total body cell potassium content would probably not have exceeded 0.5 mM per kilogram.

Calculation of the change in the potassium content of all body cells during the period of observation, as predicted from the per cent change in red cell potassium content, was made as follows:

1. The per cent change in red cell potassium content $= \dfrac{\text{Final r.b.c. potassium, in millimoles per kilogram solids, minus initial r.b.c. potassium}}{273}$

   where 273 is the mean red cell potassium per kilogram solids of 22 normal subjects.[15]

2. The calculated change in potassium content of all body cells = The per cent change in red cell potassium content (1 above) times the normal total body potassium content,

   where the "normal" total body potassium content (Ke.) in males = 43 mM × body weight in kilograms; in females = 28mM × body weight in kilograms.[16-19]

Since the patients being reported probably are potassium depleted, and since both the total body balance of potassium (A) and the change in red cell potassium, millimoles per kilogram solids (B), are referred to the mean normal Ke and red cell potassium content, respectively, the calculated per cent change of (A) and (B) will tend to be falsely low. However, no better method is known to the authors for comparing the magnitude of the change in the red cell potassium content to the change in the potassium content of other body cells using the data which are available.

The advantage of using cell solids as a reference for demonstrating a change in the cell content of electrolytes and water has been reviewed elsewhere.[15]

RESULTS

The external balances of nitrogen and potassium of 14 patients studied 23 periods are listed in Table I, and the concentration of potassium in plasma, the content of potassium in red cells, and the calculated total body cell balance of potassium of the 14 patients are presented in Table II. A change in

TABLE I. EXTERNAL BALANCES OF POTASSIUM AND NITROGEN OF 14 PATIENTS WITH SUSPECTED DISTURBANCE OF POTASSIUM METABOLISM

PATIENT	AGE	SEX	DISEASE	PERIOD	INTAKE PER PERIOD		OUTPUT PER PERIOD		BALANCE PER PERIOD	
				DAYS	POTASSIUM (mM)	NITROGEN (GM.)	POTASSIUM (mM)	NITROGEN (GM.)	POTASSIUM (mM)	NITROGEN (GM.)
1	61	M	Intestinal obstruction	0-5	320	–	481	–	–161	–
2	62	M	Intestinal obstruction	0-1	220	1.0	43	6.9	+177	–5.9
3	64	F	Vomiting	0-1	320	10.5	60	4.4	+260	+6.1
				1-2	235	9.1	197	11.6	+ 38	–2.5
4	21	F	Chronic diarrhea*	2-13	633	91.0	147	82	+486	+9
5	21	F	Chronic diarrhea*	2-14	848	101.0	318	108	+530	–7
6	15	F	Diabetic acidosis	0-1	160	–	57	–	+103	–
7	66	F	Diabetic acidosis	0-3	405	44.0	200	26	+205	+18
8	40	M	Diabetic acidosis	0-1	455	–	261	–	+194	–
9	26	F	Diabetic acidosis	0-1	117	4.1	90	11.2	+ 27	–7.1
				1-3	136	14.5	39	12.3	+ 97	+2.2
				3-5	161	24.1	106	23.9	+ 55	+0.2
				5-15	805	130.5	636	102.7	+169	+27.8
10	26	F	Diabetic acidosis	0-1	161	1.1	74	5.5	+ 87	–4.4
				1-2	141	6.7	48	5.1	+ 93	+1.6
11	46	M	Hypertension and base losing nephritis	0-1½	190	17.0	78	16.1	+112	+0.9
				1½-3	201	7.5	76	11.3	+125	–3.8
				3-5	179	8.0	98	11.3	+ 81	–3.3
				0-2	314	18.0	156	16.1	+158	+1.9
				2-3	131	3.0	138	12.8	– 7	–9.8
12	28	M	Hypertension, base losing nephritis	0-3	477	–	226	–	+251	–
13	31	M	Nephrotic syndrome	0-2	429	–	182	–	+247	–
14	43	M	Undiagnosed	0-1	426	11.9	313	11.9	+113	0

*The metabolic data of these patients have been reported earlier.[41] The days indicated here correspond to those previously reported.

TABLE II. THE INTERNAL BALANCES OF POTASSIUM, THE MEASURED TOTAL BODY CELL POTASSIUM BALANCE (A), AND THE TOTAL BODY CELL POTASSIUM BALANCE AS CALCULATED FROM THE RED CELL POTASSIUM FINDINGS (B)

PATIENT NUMBER	TIME (DAY)	WGT. (KG.)	PLASMA POTASSIUM CONCENTRATION (mM/KG.)	R.B.C. POTASSIUM PER KG. SOLIDS (mM)	THE CHANGE IN THE POTASSIUM CONTENT OF ALL CELLS AS DETERMINED BY: (A) BALANCE MEASUREMENTS (mM K)	(B) THE PER CENT CHANGE IN R.B.C. POTASSIUM CONTENT (mM K)
	0	90.9	4.2	279		
1	5	90.4	4.9	265	−148	−195
	0	54.5	3.5	223		
2	1	54.5	5.3	243	+173	+171
	0	62.4	3.3	271		
3	1	62.2	4.2	291	+232	+173
	2	62.2	6.3	294	+ 19	+ 26
					+251	+199
	0	43.4	2.2	273		
4	11	44.1	5.7	309	+430	+219
	0	44.6	2.2	269		
5	12	47.8	4.2	305	+532	+240
	0	45.4	4.8	260		
6	1	45.7	3.5	265	+115	+ 31
	0	44.0	4.8	246		
7	3	43.5	3.5	263	+216	+103
	0	50.9	2.2	197		
8	1	50.9	2.3	210	+193	+105
	0	48.0	5.5	244		
	1	48.9	3.5	249	+ 66	+ 33
9	3	48.9	5.5	266	+ 71	+115
	5	47.3	4.9	270	+ 60	+ 27
	15	46.6	3.9	276	+100	+ 39
					+297	+214
	0	46.0	3.7	246		
10	1		3.9	253	+ 97	+ 46
	2	47.0	5.1	269	+ 78	+105
					+175	+151
	0	50.0	2.8	236		
	1½	51.3	2.9	247	+100	+ 88
	3	53.6	3.8	252	+127	+ 41
	5	52.3	3.6	262	+ 92	+ 75
11					+319	+204
	0	49.1	2.3	246		
	2	48.2	3.6	264	+136	+134
	3	48.7	4.7	259	+ 9	− 38
					+145	+ 96
	0	96.9	3.0	247		
12	3	96.1	4.8	278	+216	+460
	0	63.3	3.5	208		
13	2	63.5	4.9	239	+229	+302
	0	78.0	4.4	231		
14	1	78.0	4.3	243	+115	+148

the concentration of potassium in plasma was accompanied by a similar change in the content of potassium in red cells and in the "average" body cell in 8 cases (Nos. 2, 3, 4, 5, 10, 11, 12, 13), but in acidotic diabetic Patients 6 and 7, a fall in the plasma potassium concentration and a retention of potassium in red cells and in the average body cell was observed. In columns 6 and 7 of Table II is listed the change in potassium content of all cells as estimated from balance measurements (A) and from the red cell calculation (B), as described under "Methods." In all but one instance (Case 11, second period) the changes are of similar direction and magnitude. In Patients 4 and 5 with chronic diarrhea, the red cell potassium values on admission are normal, although the serum potassium concentration, total body exchangeable potassium content, and average body cell potassium content are markedly reduced.[41] After potassium therapy, all patients, except Nos. 4 and 5, showed a percentage increase in the potassium content of red cells which was similar to that which was calculated to have occurred in the "average" body cell (Fig. 1). Patients 4 and 5, whose data appear at the extreme right of Fig. 1, had a per cent change in the potassium content of the average body cell which was about 2 times greater than that which was found in red cells.

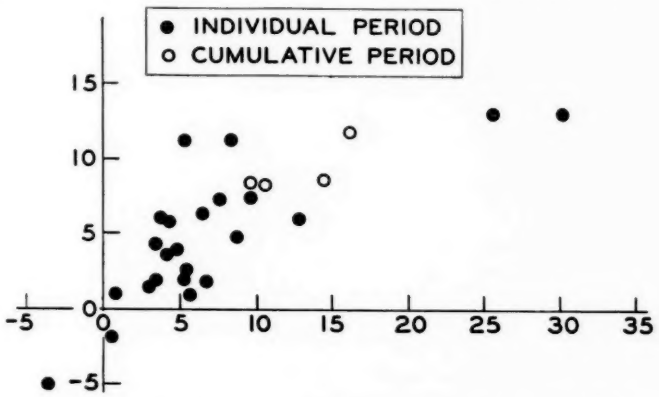

Fig. 1.—The per cent change in the potassium content of red cells (ordinate) and the total body cell (abscissa) in 14 patients with suspected disturbance in potassium metabolism.

DISCUSSION

The administration of potassium chloride to normal adults has been observed to result in the retention of less than 0.1 mM of potassium per kilogram body weight,[20] and a significant change was not found in the potassium concentration of red cells of normal subjects to whom potassium was administered.[21] Therefore an increase in the red cell potassium content and a total body retention of potassium following potassium administration suggests that a deficit of potassium was present in red cells as well as in other cells.

Previously reported data of the red cell potassium *concentration* in diabetic acidosis, gastroenteritis, and gastric alkalosis suggested the possibility that the percentile loss of potassium from red cells is similar to the loss of potassium from the average body cells. These data, which are summarized

TABLE III. RED CELL POTASSIUM DEFICIENCY IN VARIOUS DISORDERS: SUMMARY OF EARLIER DATA

AUTHORS	DIAGNOSIS	NUMBER OF CASES	DURATION OF ILLNESS (DAYS)	PER CENT DEFICIENCY* (AND RANGE) OF RED CELL POTASSIUM	PER CENT TOTAL BODY POTASSIUM DEFICIENCY	COMMENT
Kennedy, Winkley, and Dunning[2]	Gastric alkalosis	1	6 or more	29	—	
Lans, Stein, and Meyer[3]	Surgical diseases of the abdomen plus vomiting	42	?	12 (0-23)	—	Alkalosis probably present in most instances
		23	?	17	—	R.B.C. potassium returned to normal following potassium chloride administration
Eliel and Pearson[4]	Cushing's syndrome	1	100 or more	12	10.8% via balance repletion (10-15%)	Alkalosis present. 25% deficiency of muscle potassium content observed
Eliel, Pearson, and White[8]	Postoperative potassium deficit	1	11	20	10.6 via balance	Alkalosis present. 17.6% muscle potassium deficit
Eliel, Pearson, and Rawson[9]	Postoperative alkalosis	5	6-13	9.2 (0.4-19.5)	—	
Hoffman and Jacobs[11]	Vomiting	3	?	6.5	—	
Kauhtio and Hallman[5]	Gastroenteritis	28	days	15	—	Mild to severe acidosis present
Etteldorf, Hill, Tuttle, Pinheiro, Bass, and Overman[6]	Gastroenteritis	42	2-7	7 (0-20)	—	Mild to severe acidosis present
Danowski, Hald, and Peters[13]	Diabetic acidosis	5	days	21.5 (17-26.5)	—	
Guest and Rapoport[12]	Diabetic acidosis	5	days	14 (10-18)	—	
Nichols and Nichols[7]	Diabetic acidosis	9	days	15.8 (1-25)	—	

*The per cent deficiency of potassium of red cells is determined by reference to the mean normal value as determined by the author if given: otherwise the values 100 mM per liter, or 142 mM per liter of cell water are used as the normal red cell potassium concentration.

in Table III, show that the mean red cell potassium concentration is reduced from 14 to 22 per cent in diabetic acidosis, 7 to 15 per cent in gastroenteritis, and 7 to 29 per cent in alkalosis associated with vomiting. Balance studies in these disorders indicate cell potassium deficits in excess of nitrogen of similar magnitude.[22-34] The concurrently obtained red cell and metabolic balance data of Eliel, Pearson, and White,[8] and Eliel and Pearson[9] indicate that the red cell depletion of potassium during alkalosis is, if anything, greater than that of the average body cell.

Data obtained in rats have been interpreted as indicating that red cells show only to a relatively small degree the potassium depletion which occurs in muscle and possibly other cells.[35] However, since the potassium content of red cells was determined with reference to wet volume of cells and not to dry weight, and as potassium depletion was associated with alkalosis, which results in red cell dehydration, the amount of potassium lost by red cells would tend to be falsely reduced.* Wide species differences in the red cell metabolism of potassium and sodium must also be considered when one considers the results of animal experiments.[37]

The findings in Patients 4 and 5, with chronic diarrhea, of a marked reduction in the total body potassium content—not associated with alkalosis—and of red cell potassium contents which were in the normal range indicate that the potassium content of red cells and the "average" body cell does not always change equally during potassium depletion. Unequal loss of potassium from red cells and muscle cells has been reported in patients with tuberculosis,[38] and it has been shown that the potassium content of liver of potassium-deficient rats may be normal even though the skeletal muscle potassium content is markedly reduced.[39] Possibly in some disorders the loss of potassium from red cells is intermediate between muscle cells—which may regularly lose potassium during potassium depletion—and other cells which usually do not lose potassium. Another possibility is that potassium depletion of red cells depends not only on the degree or duration of total body potassium depletion but also on the presence of accompanying disorders, such as acidosis, alkalosis, and dehydration.

Since potassium deficiency of red cells has been reported only in conditions known to be associated with total body potassium deficiency, it would appear that generalized body cell potassium deficiency should be suspected if the red cell potassium content is reduced. The serum potassium concentration frequently is reduced in association with marked cell potassium deficiency, but during acidosis this may not be the case.[1, 24-26] The measurement of the red cell potassium content may therefore be especially useful in patients with hyperkalemia and acidosis. The reliability of using red cells for indicating the presence of generalized cell potassium deficiency is limited by the variability of red cell potassium content in normal subjects, in addition to unknown factors (vide supra). A similar variability of the potassium content of muscle,

*Red cells from alkalotic potassium-deficient humans may contain 8 per cent less water than normal.[36] Per liter of cells, the potassium content would therefore be higher than if referred to cell solids.

of about 10 per cent of the mean, has been found in human beings and rats.[36, 40] Therefore a 10 per cent loss of potassium from either red cells or muscle may not be detectable without prior observations, where only the normal range is available as a basis of reference. A ten per cent loss of potassium from the body, amounting to about 300 mM per 70 kilogram adult, is considered by some to be a large loss.

SUMMARY

Measurement of the red cell potassium content and of the total body balance of potassium were made concurrently in 14 subjects with suspected disturbances of potassium metabolism. Cell solids were used as a reference for quantitating changes in the red cell potassium content. In patients with acute gastroenteritis, diabetic acidosis, and base losing nephritis, the per cent change in the potassium content of red cells was similar to that of the "average" body cell, but in two chronically ill patients with laxative-induced diarrhea, the "average" body cell gained twice as much potassium as red cells. The present data, considered in light of previously reported data, are consistent with the thesis that a reduced red cell potassium content is associated with potassium depletion of the average body cell but that marked total body potassium depletion may not be associated with a reduced red cell potassium content.

We appreciate the opportunity to thank Drs. R. Berliner and E. Cotlove, National Institutes of Health, for their suggestions. Drs. W. Schwartz and A. Relman kindly supplied balance data on Patients 4 and 5, and Dr. J. McArthur supplied balance data on Patient 9.

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THE SLOW BACTERICIDAL ACTION OF TETRACYCLINE AGAINST STAPHYLOCOCCI IN BROTH

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TETRACYCLINE drugs are generally considered to be "bacteriostatic" or "essentially bacteriostatic." This interpretation of their action is based upon growth curves where bacteria are inoculated into broth containing measured concentrations of the antibiotic, but where no correction is made for the deterioration of drug.

In 1950, Bliss and associates[1] showed that when a moderately large inoculum of *Escherichia coli* was planted in tubes containing 1.5 through 80 mcg. per milliliter of oxytetracycline, the number of bacteria per milliliter diminished during the first day. With the 3 lowest concentrations used, growth reappeared at various time intervals. With the highest concentrations, a progressive diminution in the number of organisms continued until, by the end of 4 days, only 10 colonies remained; no turbidity was observed after 10 days of observation.

Hobby and associates,[2] also in 1950, demonstrated that oxytetracycline was bactericidal against very small inocula of hemolytic streptococci within 24 hours and that the rate of killing was related to the concentration of antibiotic in the medium. They stated "bactericidal action may be observed if the concentration of antibiotic is sufficiently high and the number of organisms sufficiently low." None of their experiments was carried beyond 24 hours.

In light of these observations, it seemed desirable to determine whether in broth wherein a constant concentration of the drug is maintained the initial bactericidal effect continues, or whether, after a certain percentage of the original inoculum is killed, only a bacteriostatic effect takes place. The action of tetracycline was accordingly studied against 4 strains of Staphylococcus recovered from cases of bacterial endocarditis. Constant levels of the antibiotic were maintained. The results demonstrate that in yeast beef broth, tetracycline exerts a slow bactericidal effect and that the rate of bactericidal action is a function of the concentration of drug in the media.

METHODS

1. *Inoculation of Broth.*—Experiments were carried out in Erlenmeyer flasks containing a total of 50 ml. of yeast beef broth (Penassay broth, Difco). One milliliter of a 16-hour culture, either undiluted or diluted in broth to 10^{-3} or 10^{-6}, was inoculated into the 48

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ml. in the flask. One milliliter of concentrated tetracycline* solution was then added. The flasks were then incubated at 37° C. until at least 2 negative subcultures were obtained.

2. *Subcultures.*—Subcultures were made daily. During the first few days of the experiment, one milliliter was removed and appropriate tenfold dilutions prepared, from which pour plates were made for the purpose of counting. As the number of organisms in the flask declined, one milliliter of undiluted culture or of a 10^{-1} dilution was plated. Inasmuch as we were interested in absolute sterility, a problem arose when the amount of tetracycline transferred from the flask to the agar plate was sufficient to inhibit the growth of any staphylococci which might be present. In order to overcome this, advantage was taken of the discovery of Albert,[3, 4] Weinberg,[5] and others[6, 7] that magnesium sulfate exerts an antagonistic effect upon tetracycline drugs. Investigation of this phenomenon, as it applied to our own problem, demonstrated that 10 to 100 mg. of magnesium sulfate (C.P.) per milliliter of agar antagonized 0.25 to 5 mcg. per milliliter of tetracycline.[8] Colonies appeared in the $MgSO_4$ plates when no colonies appeared in identical plates to which no $MgSO_4$ had been added.

In some of the experiments, where the aim was the determination of the number of days required to sterilize the culture rather than the construction of a bactericidal curve, subcultures were made in broth containing $MgSO_4$, rather than in pour plates.

3. *Criteria of Sterilization.*—

A. Negative subcultures when $MgSO_4$ was added to the medium.

B. When at least 2 negative subcultures were obtained, the Erlenmeyer flask was covered with Parafilm or aluminum foil to prevent evaporation of broth, and returned to the incubator. The level of tetracycline was measured once or twice a week until the concentration of the drug fell below the minimal inhibitory concentration (M.I.C.). At this time the following procedures were carried out: (1) Ten milliliters of the old culture was centrifuged and the supernatant discarded. A gram stain was made of the sediment. Two loopfuls of the sediment were subcultured into fresh broth; all these cultures were negative. (2) Ten milliliters of the remaining old broth was subcultured into 50 ml. of fresh broth. These cultures were uniformly negative. (3) As soon as the negative subculture was established, another aliquot of the old broth was seeded with a small inoculum of staphylococci to see whether it would support growth. Rapid growth occurred in every instance.

4. *Maintenance of Tetracycline Level.*—The level of tetracycline in the flask was determined daily by twofold serial dilution or measured dilution tests, with use of *Bacillus cereus* as the test organism. The concentration sequence of the measured dilution test was, for the 50 mcg. flask, 15, 20, 22.2, 28.5, 33.3, 40, 50, 66.6, 75, and 100. For the 2.5 mcg. per milliliter flask it was 1.0, 1.2, 1.4, 1.6, 2.0, 2.5, 3.3, and 5. Additional tetracycline dissolved in yeast beef broth was added each day, the amount being estimated from the previous day's level. Usually between 50 and 80 per cent of the original quantity of drug in the flask was added each day while the experiment was in progress. After 2 to 4 negative subcultures were obtained no further tetracycline was added.

RESULTS

Minimal Inhibiting Concentration of the Strains Studied.—The minimal inhibiting concentrations (M.I.C.) of the 4 strains were determined by a twofold serial dilution test in yeast beef broth, and customary bacteriostatic end points read after 18 hours' incubation at 37° C. The M.I.C. for 2 of the strains was 0.12 mcg. per milliliter, and for the other two 0.25 (Table I). The initial bacterial density in these tests approximated that of the bactericidal tests reported below where undiluted culture was inoculated.

*The tetracycline was Achromycin, supplied through the courtesy of Dr. S. M. Hardy of the Lederle Laboratories.

TABLE I. MINIMAL INHIBITING CONCENTRATIONS (M.I.C.) OF TETRACYCLINE FOR FOUR STRAINS OF STAPHYLOCOCCI

STRAIN	DESCRIPTION	M.I.C. MCG. PER ML.
W.E.	*S. albus*, non-hem.	0.12
J.C.	*S. aureus*, hem.	0.12
H.H.	*S. aureus*, hem.	0.25
W.V.	*S. aureus*, hem.	0.25

Sterilization of Yeast Beef Broth Cultures by Tetracycline.—The summarized results are presented in Table II. In most of the experiments, one high concentration of tetracycline, namely 50 mcg. per milliliter, and one low concentration, namely 2.5 mcg. per milliliter, were studied. These concentrations were approximately 300 and 15 times the M.I.C. In a few others the effect of 25, 10, or 5 mcg. per milliliter was also investigated. In most of the tests, undiluted culture was employed, resulting in an initial population density of 10^7 or 10^6 after the inoculum was diluted in the 50 ml. of broth. The effect of the same concentrations of antibiotic upon inocula diluted to 10^{-3} and 10^{-6} was also investigated.

TABLE II. AVERAGE NUMBER OF DAYS REQUIRED BY TETRACYCLINE TO ACHIEVE COMPLETE STERILIZATION OF YEAST BEEF BROTH CULTURES OF STAPHYLOCOCCI

STRAIN	INITIAL POPULATION DENSITY, BACTERIA PER ML.*	TETRACYCLINE CONCENTRATION (MCG. PER ML.)			
		50	*10*	*5*	*2.5*
H.H.	10^7-10^8	3.3	8		10
W.E.	10^7-10^8	3.7			6.7
W.V.	10^7-10^8	4	5		14
J.C.	10^8-10^9	3.6		12	9.3
H.H.	10^4-10^5	3	4		5
W.E.	10^4-10^5	1	3		5
H.H.	10 -10^2	1	2		1
W.E.	10 -10^2	1	1		1

*As determined by pour plates before the inoculum was added to the Erlenmeyer flask. The bacterial density in the flasks was one-fiftieth that given in this column.

With all 4 strains, 50 mcg. per milliliter sterilized the large inoculum in 3 to 5 days. When lower concentrations of tetracycline were employed, longer incubation periods were needed to produce sterilization, and the killing times were more variable. Thus, the W.E. strain was sterilized in an average of 6.7 days by 2.5 mcg. per milliliter of tetracycline, whereas the W.V. strain required 14.

The smaller the initial population density, the more rapidly was the flask sterilized. In most instances flasks inoculated with 10 to 25 cells (10^{-6} dilution of the original culture) were sterile at the end of 24 hours. In Fig. 1 the bactericidal curves of 50, 10, and 2.5 mcg. per milliliter against a large inoculum of the H.H. strain are plotted. It is seen that the initial slope is steeper with the higher concentrations of drugs, and the number of days required to produce complete sterilization shorter.

Appearance of Staphylococcal Cells at Termination of Bactericidal Period.—A gram stain of the centrifuged sediment of broth at the end of an experiment showed a few disorganized bacterial cells. Some were gram negative. They were never of normal size, being smaller or larger than typical staphylococci. Most of them were misshapen and some had the appearance of large, hollow shells. In contrast, cells from control flasks incubated as long as 7 weeks had the appearance of normal healthy staphylococci.

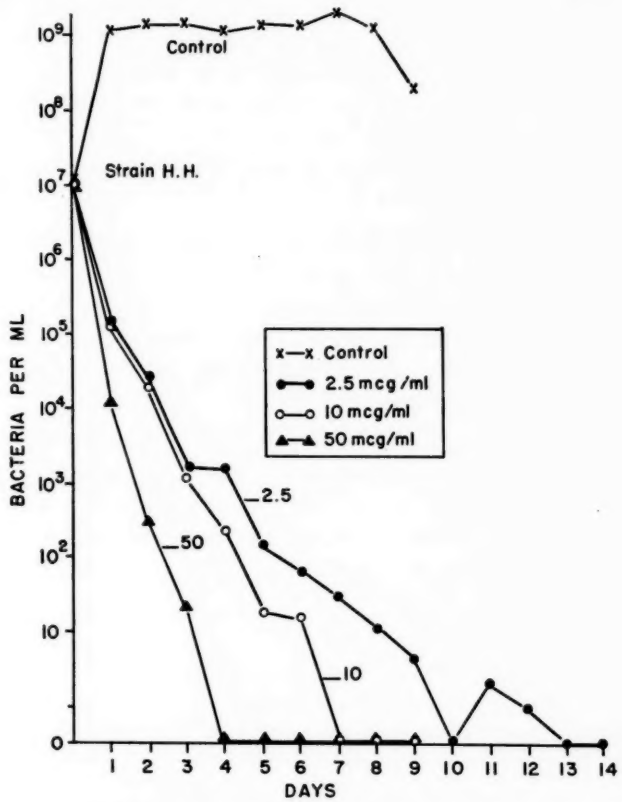

Fig. 1—The slow bactericidal action of tetracycline against staphylococcus in yeast beef broth.

DISCUSSION

These data demonstrate that tetracycline produces a slow bactericidal action against strains of pathogenic staphylococci in yeast beef broth when the concentration of the antibiotic is maintained at a reasonably constant level. Though it was recognized by Hobby in 1950 that bactericidal action may be observed "if the concentration of antibiotic is sufficiently high and the number of organisms sufficiently low," it has not been previously demonstrated that in broth such action occurs with large inocula of organisms and with relatively low concentration of drug.

We should like to suggest that the definitions of bactericidal and bacteriostatic action be modified with profit to those who read the literature of

antimicrobial chemotherapy. Davis and McDermott[9] define a bacteriostatic agent as "one whose presence, in a culture that would otherwise be growing, stops growth; as a result the number of viable organisms remains constant for hours or even days. . . . A bactericidal agent in contrast, causes a progressive decrease in the number of viable organisms which cannot be reversed by dilution or by addition of any available substances." In an excellent discussion of bactericidal and bacteriostatic agents, they then explore the implications of these definitions and exceptions to them. In a later paragraph they state, "despite these qualifications, it is thoroughly useful to distinguish between a bacteriostatic drug, which produces a constant viable count for at least several hours, and a bactericidal one, which causes a rapid decline in the viable count. It makes a great difference, whether after a limited period of exposure to a drug, growth is initiated by most of the bacteria initially present or by only a tiny fraction of them."

If we consider the action of antimicrobial drugs against large inocula of bacteria in terms of days rather than hours, a simple and generally informative classification might be (1) bacteriostatic drugs, (2) slowly bactericidal drugs, and (3) rapidly bactericidal drugs. Examples of bacteriostatic drugs would be sulfonamides; of slowly bactericidal drugs, tetracycline; and rapidly bactericidal drugs, penicillin and streptomycin.

Much remains to be done, however, before any tentative classification of antimicrobial drugs based on in vitro studies can be applied to their action in mammalian tissues. The problem of "persisters," the problem of the slower action of chemotherapeutic drugs in blood clots than in broth, as shown by Hunter,*[10] and the role of the host's tissues and body fluids all have a bearing upon the main issue.

The importance of initial bacterial density and its relation to the likelihood of bactericidal action, along with the significance of drug concentraton, raises the question as to whether slowly bactericidal drugs could have a place in chemoprophylaxis, where the destruction of small numbers of bacteria is important.

SUMMARY

Tetracycline has a slow bactericidal action in yeast beef broth against staphylococci recovered from cases of bacterial endocarditis, provided a constant concentration of the antibiotic is maintained. When large inocula are planted, the number of staphylococcus cells recovered from the broth diminishes fairly rapidly over a period of a few days. With a concentration of tetracycline 300 times the minimal inhibiting concentration (M.I.C.), i.e., 50 mcg. per milliliter, sterilization is completed in 3 to 5 days. With a concentration about 15 times the M.I.C., i.e., 2.5 mcg. per milliliter, sterilization proceeds much more slowly. When moderate or small numbers of staphylococci are inoculated, the bactericidal rate is correspondingly more rapid, but still proportional to the concentration of tetracycline.

*We have been able to confirm this observation for penicillin and for tetracycline.

Rigid criteria of sterilization were established. In cultures where all efforts to demonstrate the presence of viable cells failed, gram-stained sediments contained distorted cell bodies.

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THE THERAPEUTIC EXPERIMENT: OBSERVATIONS ON THE MEANING OF CONTROLS AND ON BIOLOGIC VARIATION RESULTING FROM THE TREATMENT SITUATION

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RECENTLY, the need for placebo controls in therapeutic experiments has been questioned and it has even been said that data from placebo trials may be misleading.[1, 2] It has thus been implied that the effects of a pharmacodynamic agent may be appraised by comparison of a "treated" with an "untreated" group of subjects. The present report is based on experience with the attempt to control ipecac-induced nausea and vomiting. It provides evidence that the treatment situation itself may introduce a vast and significant variation into a previously stable and uniform body of data observed in a control (untreated) group of human subjects.

PROCEDURE

Six ml. of syrup of ipecac was administered by mouth to 26 healthy human subjects. In 4 additional series of experiments at weekly intervals, these same 26 subjects swallowed a capsule 2 hours prior to the administration of ipecac. The contents of the capsules were not know to either experimenter or subject. Both knew, however, that an antiemetic effect was being sought. Each capsule contained one of the following: (1) chlorpromazine, 20 mg. (drug A), (2) SKF 4657-I_2 (2-choro-10-[3-(N-Methylpiperzinyl)-Propyl]-Phenothiazine Dimaleate), 20 mg. (drug B), or (3) placebo drug P). The capsules were assigned to the various subjects for the 4 trials according to a systematic random sampling technique. At the conclusion of these experiments and again with an interval of one week between each observation on the group of subjects, the entire series was repeated using 4 ml. of ipecac. On an additional and final week, the individuals were given 5 ml. of a simulated (placebo) syrup of ipecac without prior medication.

RESULTS

No significant difference was observed in the incidence, the time of onset, or the duration of nausea and vomiting between the untreated control groups given 6 ml. and 4 ml. of ipecac. Nausea was noted on every occasion, and vomiting with equal frequency in the two groups, despite the possible suggestive effect of reducing the dose of the emetic stimulus from 6 to 4 ml. In contrast, the placebo syrup of ipecac induced significantly less nausea and no vomiting, despite the possible suggestive effect of increasing the dose from 4 to 5 ml. (Fig. 1). These data establish that the nausea and vomiting were primarily

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chemically and not psychically induced, and that the 4 ml. dose of syrup of ipecac apparently was as potent a stimulus for inducing nausea and vomiting as was the 6 ml. dose.

When ipecac ingestion was preceded by one of the test capsules, the previously uniformly nauseating effects of ipecac no longer obtained. The various capsules reduced the incidence of nausea to as low as 83 per cent and of vomiting to as low as 57 per cent. In all instances the differences from the

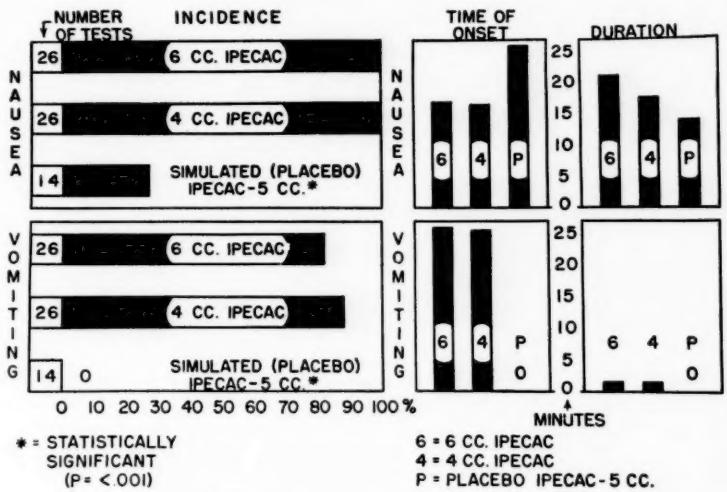

Fig. 1.—The incidence, the time of onset, and the duration of nausea and vomiting in the "untreated" control ipecac tests compared with the "untreated" simulated (placebo) ipecac tests.

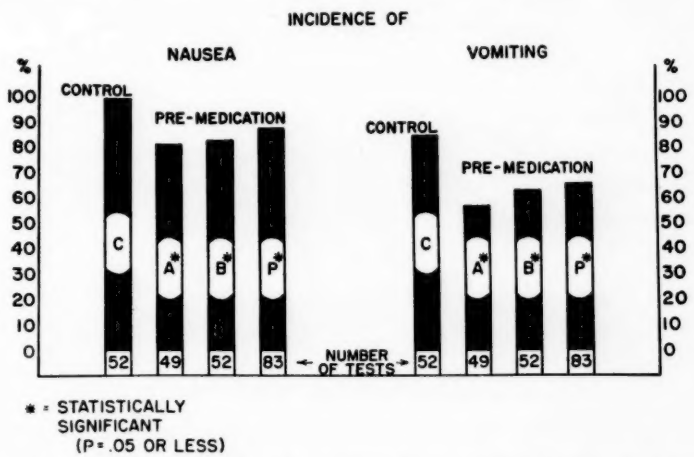

Fig. 2.—The incidence of nausea and vomiting in the "untreated" control ipecac tests (*C*) compared with the "treated" ipecac tests with drug *A*, drug *B*, and with placebo (*P*) as premedication.

control data were statistically significant at the level of $p = 0.05$ or less. Neither of the drugs tested afforded more consistent or more significant protection than did the placebo (Fig. 2).

These observations illustrate in vivid fashion the variation which may be introduced into a previously stable biologic pattern by a treatment situation. Following ingestion of ipecac alone on 2 separate trials (52 observations in 26 individuals), the incidence of nausea was 100 per cent, and of vomiting 85 per cent. Premedication with capsules blindly and randomly administered altered this previously uniform pattern so that the incidence of nausea was no longer 100 per cent but now varied from 82 to 88 per cent. The incidence of vomiting now varied from 57 to 66 per cent. These variations could not be attributed to the pharmacodynamic properties of the various agents since the placebo did as well and as poorly as either of the drugs tested. Moreover, in 2 different trials with the same agent in the same individual, the protection afforded was equally inconsistent whether the agent was placebo or one of the drugs (Fig. 3). The incidence and inconsistency of the protection responses

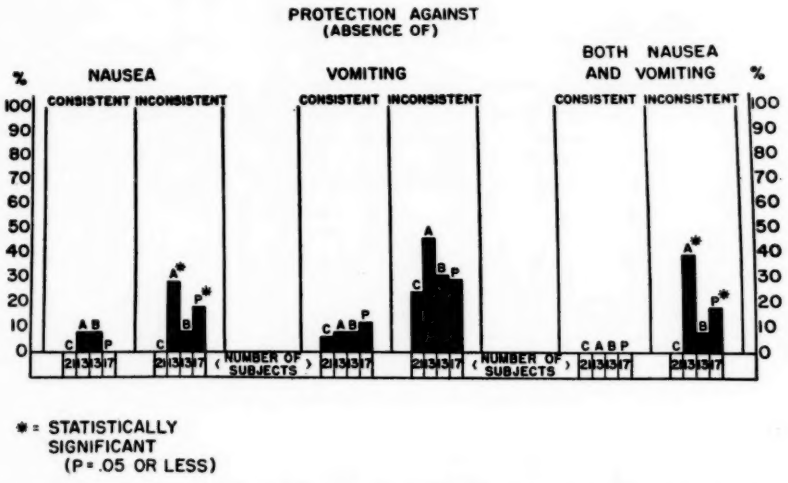

Fig. 3.—The incidence of consistent and inconsistent complete protection responses (absence of nausea and/or vomiting) observed in two identical tests in the same individual. The only significant protection found was inconsistent. *C* = individuals with two "untreated" control ipecac tests. *A* and *B* = individuals with two "treated" ipecac tests with the same premedication, drug *A* or drug *B*, respectively. *P* = individuals with two "treated" ipecac tests with placebo as premedication.

observed with prior placebo medication were virtually the same with the 6 ml. and the 4 ml. doses of syrup of ipecac. This, together with the fact that without prior medication the responses of the same subjects to the 6 ml. dose of ipecac were indistinguishable from those observed when 4 ml. was given, strongly supports the implication that the variations observed were not due to a difference in the size of the dose of ipecac but rather to the fact that individuals respond inconsistently to the placebo. These findings do not support the concept of a placebo "reactor," who would be expected to respond in a consistent fashion to a placebo medication. A more detailed consideration of this aspect of the data is the subject of a separate report,[3] which contains further observations on the placebo "reactor" and "non-reactor."

Data were also gathered on each individual's subjective estimate of the comparative severity of nausea and vomiting on each occasion. The experimental subjects noted no mitigation of the experience when the dose of ipecac was reduced from 6 ml. to 4 ml., but on the occasion of premedication with capsules they claimed there was a uniform and substantial lessening of discomfort. Thus in an uncontrolled therapeutic trial the 50 to 65 per cent "satisfactory" protection against nausea and vomiting afforded by these agents would be considered clinically significant at the level of $p = 0.01$ or less. Only the inclusion of the placebo data prevented the erroneous conclusion that a specific pharmacodynamic effect had been demonstrated (Fig. 4).

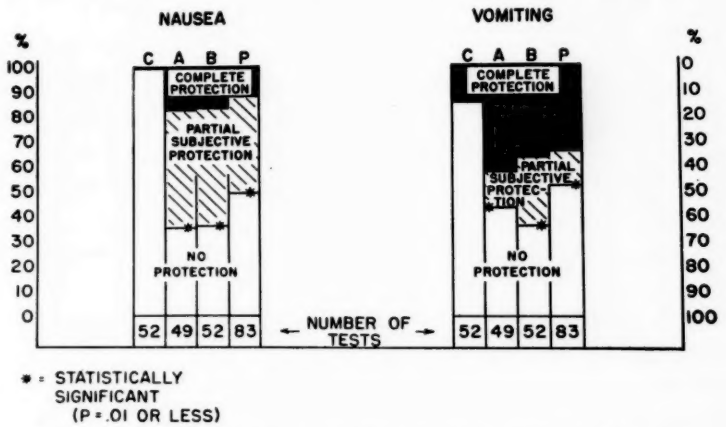

Fig. 4.—The incidence of complete protection, partial subjective protection, and no protection against nausea and vomiting observed in the "untreated" control ipecac tests (*C*) compared with the "treated" ipecac tests with drug *A*, drug *B*, and with placebo (*P*) as premedication.

CONCLUSION

Without allowing for any inference regarding the specific pharmacodynamic effects of the agent or agents tested, one may assume that a relatively uniform and predictable chemically-induced bodily reaction (nausea and vomiting from syrup of ipecac) may be caused to vary to a statistically significant degree by drug "treatment." These findings emphasize the urgent need for the blind and random placebo control in therapeutic research.

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LABORATORY METHODS

NEUTRON ACTIVATION ANALYSIS OF TISSUE: MEASUREMENTS OF SODIUM, POTASSIUM, AND PHOSPHORUS IN MUSCLE

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IN MANY current clinical investigations, it is desirable to obtain information on electrolyte concentration in tissue. Such information is even more valuable when serial studies can be made in individual cases. However, the size of sample required for analysis by ordinary chemical or flame photometric methods is a serious deterrent to the performance of such measurements,† prohibiting the use of small tissue samples obtained with a biopsy needle. Since recent developments in nuclear physics, especially in neutron activation techniques,[1] would permit in principle, quantitative chemical analysis of exceedingly small samples, it was deemed desirable to explore the use of this method for the analysis of electrolytes in tissue.

Neutron activation analysis is essentially a two-step process. The first step involves the production of the radioactivity within the sample, and the second involves the selective detection of radiations from the desired elements. Because of the high neutron fluxes available from modern nuclear reactors and because of the extreme sensitivity of radiation detectors, the method can offer millimicrogram sensitivity, or better. In the present work the method has been successfully applied to the determination of sodium, potassium, and phosphorus in milligram samples of human muscle tissue. This work was undertaken in order to assess the reliability and sensitivity of the neutron activation analysis and to establish its practicality in comparison with other more usual analytic methods.

METHODS

For clinical reasons, it was necessary to use relatively small amounts of tissue. Consequently, to facilitate handling, the neutron bombardment of the sample had to take place within suitable containers. Since it was desirable not to use carrier-chemical methods

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†See, however, A. K. Solomon, Anal. Chem. **27**: 1849, 1955, for a specialized method of flame photometry with high sensitivity.

to separate the radioactivities, a search was made for activation containers from which the tissue need not be removed during the measurement process. This implied that the radiometric methods of discrimination, to be described subsequently, must not encounter interference from radiation from the containers. Three container materials were investigated in detail: spectroscopically pure aluminum (99.999 per cent), triplydistilled styrene (polymerized in quartz by ultraviolet radiation), and the reactor grade graphite (grade AGOT).* Since both the plastic and the aluminum produced interfering gamma radiations, they were not satisfactory. Although the reactor grade graphite exhibited a very small amount of gamma ray activity and some soft beta activity, it was found to be quite satisfactory. In consequence, small screw-cap cups were fabricated for use as irradiation containers for the tissue samples. The design of the irradiation container is shown in Fig. 1.

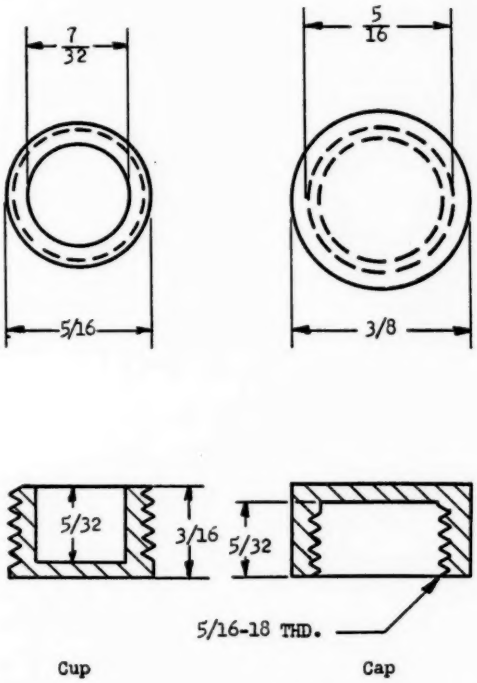

Fig. 1.—Sketch of irradiation container. Material is reactor-grade graphite. All dimensions in inches.

To maintain the low activity inherent in the graphite, we had to prevent skin contact during the machining of the container and during any subsequent manipulations prior to irradiation. Therefore, acid-washed rubber gloves and acid-washed equipment were used during manipulation of the tissue and containers.

The skeletal muscle specimens used in the first experiments were obtained from the deltoid or gastrocnemius muscles, under local (one per cent procaine) anesthesia, or from the pectoralis major, during operations on the breast. Later, samples were taken with a Bickel biopsy needle. Care was taken to avoid infiltration of the muscle with the anesthetic agent. On removal, the sample was transferred to an acid-washed plastic slab and dissected free of gross fat and fibrous tissue. When the sample was loaded into the graphite container,† whose weight was established, the wet weight of the tissue was determined by

*Graphite supplied by Argonne National Laboratories.

†In many cases, the tissue samples were stored in glass in a Deepfreeze until a number had accumulated, and then were placed in the graphite just prior to drying, weighing, and irradiation.

differences. The graphite cup was placed in an oven at 100° C. for about 20 hours, until constant weight had been reached, enabling dry weight to be calculated by differences. At this point the container was sealed with its screw cap, wrapped in Pliofilm, and inserted in an aluminum irradiation tube for reactor bombardment. As many as twenty samples can be handled in one irradiation tube. Since the neutron flux and absorption cross sections for some elements are not well established, it is most satisfactory in an activation analysis procedure to incorporate standards for the elements of interest within the same irradiation tube used for the samples themselves. Suitable standards were prepared from solutions of distilled water and reagent grade KCl, NaCl, and KH_2PO_4. Measured volumes of solutions of known concentration were pipetted into their own graphite containers and irradiated along with the tissue samples.

At the conclusion of a 12-hour neutron bombardment* at a flux of approximately 1.3×10^{13} neutrons/cm.^{2}/sec., the samples and standards were removed from the irradiation container, and quantitative determination of the induced activities was made. Carrier-chemical methods were avoided not only because of the greater effort involved and because of the destructive nature of the technique, but also because purely radiometric methods would be nondestructive. Radiation damage effects were, of course, inevitable, but elemental chemical composition was unaltered.

Since in a 12-hour bombardment of muscle tissue, only sodium, potassium, and phosphorus contributed significantly to the activity that resulted, it was necessary to find a method to discriminate between radiations from these three elements. Table I lists half-lives and radiation energies for the neutron-produced radioactive isotopes of the three elements in question. Both of the beta rays resulting from the decay of potassium have higher energy than the betas from either of the other two elements. This suggested the determination of potassium by counting only those betas with energy greater than 1.75 mev. Similarly, the 2.76 mev. gamma ray from sodium is well separated from all the other gammas and can be counted in a scintillation spectrometer. Since phosphorus, however, emits only an intermediate energy beta, its radiometric separation had to be approached differently. It is evident from Table I that P^{32} has a much longer half-life than the other two important isotopes; a simple calculation shows that an aging time of eight days will reduce the P^{32} activity less than a factor of two, while the other activities decrease by a factor of more than 5,000. A suitable delay, then, eliminates all interference in a phosphorus activity determination on the muscle tissue. Although rather elaborate spectrometer equipment was first used for these analyses, it was found possible to simplify the counting procedure to one employing only a simple Geiger counter and scaler. The simplified procedure is as follows: The ratio of gamma to gamma plus beta counting rate for potassium was obtained from the standard made up from KCl solution using an end-window Geiger counter and aluminum absorbers corresponding to 1.7 Gm. per square centimeter and 0.8 Gm. per square centimeter. The ratio of counting rates for the sodium standard was obtained with the same two absorbers. The 0.8 Gm. per square centimeter absorber is sufficient to stop all betas of less than 1.75 mev., while the thicker absorber will stop betas of energy less than 3.8 mev., which includes

*Bombardments were made in Argonne National Laboratories' CP-5 reactor. This is a heavy water moderated reactor using aluminum-clad enriched fuel and operating at a power level of two megawatts. An irradiation tube containing twenty samples may be processed at a cost of approximately 25 dollars.

TABLE I. NUCLEAR CHARACTERISTICS OF SODIUM POTASSIUM AND PHOSPHORUS

ELEMENT	ISOTOPE	HALF-LIFE	BETA ENERGY	GAMMA ENERGY
Potassium	K^{42}	12.5 hours	3.58 mev. 2.04	1.5 mev.
Sodium	Na^{24}	15 hours	1.4 mev.	1.37
Phosphorus	P^{32}	14.3 days	1.7	---

all betas from the elements of interest. The gamma ray attenuation, of course, will be only very slight. From counting rate data on the tissue samples themselves taken with the two absorbers, it is then possible to calculate the sodium and potassium content by successive approximations. All counting is done, of course, in a fixed geometry with open containers and with suitable corrections for decay and coincidence losses.

Assuming the counting rate obtained with the thick absorber is due entirely to sodium, the sodium counting rate with the thinner absorber can be calculated from the previously determined sodium ratio. A subtraction then gives an approximate potassium contribution with the thinner absorber. Using the known potassium ratio, one can calculate the potassium contribution with the thick absorber. The original counting rate (assumed to be due to sodium) then is reduced by the approximate potassium contribution, and the entire process is repeated. Since the calculation converges very rapidly, two repeats are sufficient to reduce to less than one per cent the error due to the approximation. The phosphorus level is determined by measuring the beta activity of the sample after a delay of 8 to 14 days. In all cases, counting rate is converted to mass by comparison of the standards and the isolated sample activities.

RESULTS

As a first test of the method, a large group of standard solutions of sodium and potassium chloride was analyzed. A maximum deviation of about 5 per cent obtained in the results of this test was attributable in great measure to limitations on available pipetting procedures for handling the rather small liquid volumes. Contributions to this spread also may have arisen from nonuniform distributions of solution residue within the counting cups. The actual counting data are shown in Table II.

TABLE II. COUNTING RATES FOR STANDARD SOLUTIONS OF SODIUM AND POTASSIUM CHLORIDE

STANDARD	COUNTS/MG./SEC.	DEVIATION FROM AVERAGE (IN PER CENT)	COUNTS/MG./SEC.	DEVIATION FROM AVERAGE (IN PER CENT)
A	193,000	+3.1	24,000	+2.8
B	191,000	+2.0	22,400	−4.0
C	195,500	+4.5	23,150	−0.9
D	185,000	−1.2	23,100	−1.1
E	180,800	−3.4	24,050	+3.0
F	177,700	−5.1	23,400	+0.2
Average	187,200		23,350	

A further check on the reliability of the method was made with combined tissue and standard solution samples. Twelve tissue samples from the same muscle were obtained. Six of these samples were prepared with added sodium, potassium, or sodium-potassium solutions. These samples, together with the remaining six tissue samples, were irradiated along with a set of standards. From the average values of sodium and potassium obtained from the untreated tissue, the quantity of additive in the treated samples was determined to within 10 per cent accuracy, as shown in Table III.

TABLE III. RESULTS OF ADDITIVITY AND LINEARITY WHEN 50 LAMBDA STANDARD SOLUTIONS OF NaCl AND KCl WERE ADDED TO TISSUE SAMPLES CONTAINING KNOWN AMOUNTS OF Na AND K

LAMBDA OF STANDARD SOLUTIONS ADDED TO TISSUE*		ADDITION INFERRED FROM COUNTING DATA	
Na	K	Na	K
50	--	55	--
50	--	54	--
--	50	--	53
--	50	--	56
50	50	53	48

*Tissue mean values: Na = 302 mg./100 Gm. (dry weight).
K = 1443 mg./100 Gm. (dry weight).

Since these measurements were based on average values rather than on specific samples, the results were deemed an adequate confirmation of the additivity and linearity of the method. With confidence in the method established, 19 samples obtained from both normal and pathologic tissue were analyzed. These samples ranged in dry weight from 1.7 to 15.5 mg. Since counting rates ranged upward from a minimum of 1,000 counts per minute, because of phosphorus contained in the smallest samples, adequate data could be obtained with 0.1 mg. samples. Further, the neutron flux could be increased if desired to allow determinations on samples as small as 10 μg. The significance of differences between normal and pathologic tissues, as shown in these measurements, is discussed elsewhere.[2, 3]

DISCUSSION

The results obtained by this method are essentially in agreement with those obtained by more conventional methods using larger sample sizes. The accuracy of the analysis, of the order of 5 per cent, is limited by errors in the weighing and pipetting procedures used in the preparation of samples and standards. The required measurements for sodium, potassium, and phosphorus determinations on milligram and submilligram quantities of fresh tissue are made with a very simple counting arrangement consisting of an end-window Geiger counter, two aluminum absorbers, and a combination high voltage supply and scaling unit. In general, the levels of activity are sufficiently high so that the analysis may be made as long as two or three days after the neutron irradiation. This reduces the requirement of proximity to a reactor to the point where samples can be mailed to a suitable reactor facility, irradiated at a cost of about 25 dollars for twenty samples, and returned by air

mail to any laboratory desiring to use the technique. Since the measurements themselves are perfectly straightforward, approximately 20 minutes is required to obtain the counting data for a sodium and potassium determination, with another few minutes after an aging period to provide for phosphorus determinations.

CONCLUSION

The suitability of neutron activation analysis methods to the determination of sodium, potassium, and phosphorus in milligram or submilligram samples of tissue has been established. The method is nondestructive in an elemental chemical sense and is accurate to approximately 5 per cent. The required equipment, which is exceedingly simple, may be found in any laboratory equipped for tracer studies. The nuclear parameters and induced activity levels are such that mail service to a reactor will suffice for irradiation.

We wish to thank Drs. Sol Rosenthal, Robert M. Kark, John D. Williams, and Barbara Ansell for their help and advice.

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A METHOD OF PLASMA DIALYSIS AND ITS APPLICATION TO THE DETERMINATION OF THE DIFFUSIBLE FRACTION OF CALCIUM

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ELIMINATION of a solute in the glomeruler filtrate and its distribution between vascular and interstitial fluid compartments of the body are both restricted if the solute is bound by the plasma proteins. Measurement of the diffusible fraction is therefore necessary in studies describing either distribution or secretion of such substances. A simple and convenient system of compensated dialysis, useful in the determination of free and bound fractions, is described below.

Roma and Takahashi[1] observed some years ago that only a portion of the calcium of serum is diffusible. Subsequently both total[2] and diffusible[3] serum calcium concentrations were shown to be functions of serum protein content. Ionizable calcium has likewise been related to protein concentration.[4] Some[5, 6] maintain that the diffusible fraction of calcium is an inverse function of plasma pH; others[7-9] claim that these variables are unrelated. Finally, it has been claimed that, in vivo, the unfilterable fraction decreases as the concentration of calcium in the serum is raised. On the other hand the addition of calcium to serum in vitro has been described as causing little or no alteration in the fraction bound.[10]

In a study of the effects of these several variables on the binding of calcium, we have observed that the dialyzable fraction varies with pH, calcium concentration, and protein concentration of the plasma. A quantitative description, undertaken as a preliminary step in the study of the mechanism of excretion and reabsorption of calcium by the kidney, is presented below.

METHOD OF PLASMA DIALYSIS

The equipment for dialysis, described below, was evolved from a more complicated apparatus used in the study of the plasma binding of phenol red. It has the virtue that the individual dialysis units are inexpensive and simple to clean after use, and that they require a sample of only 4 ml. of plasma, have a high surface to volume ratio, and permit exact control of temperature and pH during equilibration.

The components of and mode of assembly of a single dialysis unit are shown in Fig. 1, *A*. Charging, final assembly, and disassembly, are shown in Fig. 1, *B*. The basic elements are a glass tube, flared at both ends (part 13), 12 mm. in outside diameter by 22 cm. in length with a wall 1 mm. in thickness; and Visking seamless cellulose tubing, ¼ inch in inflated diameter (part 5).

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Construction of Lower Unit.—A 15 gauge intravenous needle (part 2) is forced through the center of a No. 00 rubber stopper (part 1), the top one-third of which has been cut off with a razor blade. This needle serves as a guide to the introduction of a cannula of slightly greater diameter (part 3) through the stopper from the opposite direction. The twisted end of a 30 cm. length of Visking tubing is inserted into the lumen and the cannula withdrawn. The stopper provides a leak-proof closure of the end of the tubing. However, it is wise to test each unit with air pressure and immersion in water before use. The Visking tubing is then inserted in the glass tube, the rubber stopper forced home, and the lower end of the unit sealed by dipping in hot paraffin.

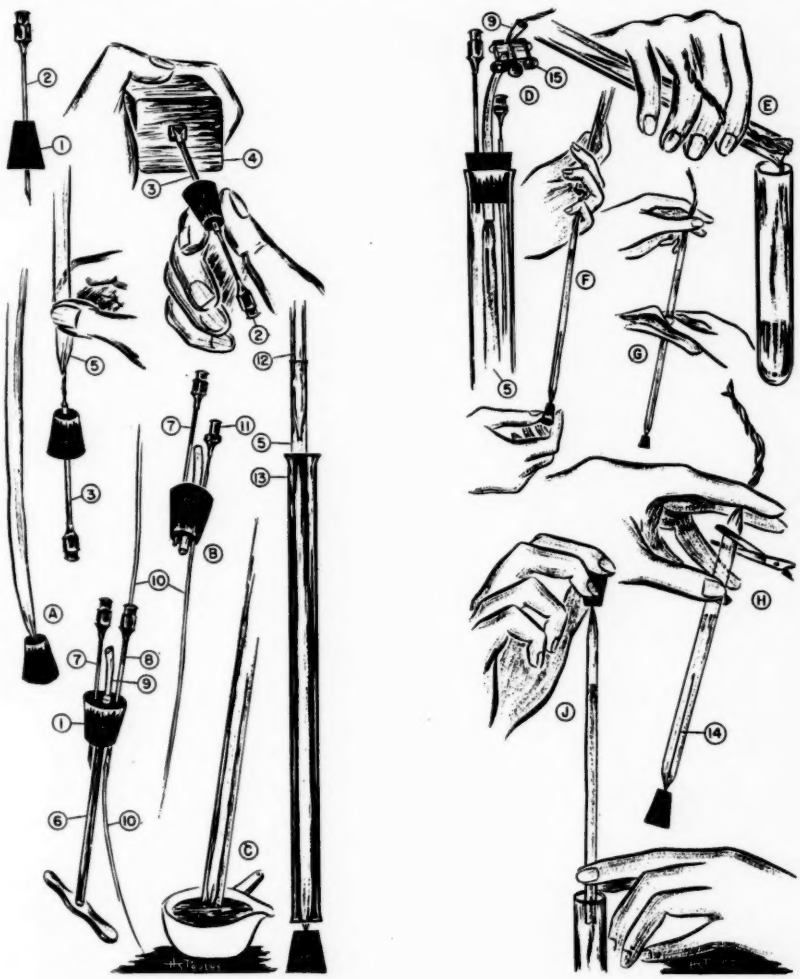

Fig. 1.—*A*, Components of and mode of assembly of a dialysis unit. *B*, Mode of disassembly of a unit. For description, see text.

Construction of Upper Unit.—The upper end of the unit is formed from a No. 00 rubber stopper similar to that used for the bottom. It passes three elements: a piece of ⅛ inch polyethylene tubing 5 cm. long, an 18 gauge needle for the exit of gas and a 20 cm. length of polyethylene tubing, 0.038 inch in outside diameter, for the introduction of gas. The ⅛ inch polyethylene tubing (part 9) is introduced through a cork

borer (part 6), as shown in the lower left side of Fig. 1, *A*. The cork borer is then withdrawn. The small polyethylene tubing (part 10) is introduced through a 17 gauge needle (part 8) and the needle withdrawn. To connect the gas inflow, we inserted a 25 gauge needle (Part 11) into the upper end of the small polyethylene tubing. Roughly 20 cm. of this small tubing extends below the stopper. It reaches to the bottom of the glass tube and the continuous bubbling of gas serves to mix the dialyzing solution. It is convenient to prepare a number of upper and lower units as stock items for charging and assembly as needed.

Charging and Assembly of Units.—The dialyzing unit has been designed to hold 4 ml. of plasma inside the Visking tubing and 6 ml. of buffered, isotonic, balanced salt solution oustide, i.e., between the dialyzing membrane and the glass tube. A mixing rod (part 14) is placed inside the dialyzing tubing. The twisted upper end of this tubing is inserted through the large polyethylene tube and a screw clamp (part 15) is applied and tightened. The unit is connected as shown in Fig. 2. A vacuum line is attached to the 18 gauge needle which serves for gas outflow. A CO_2-O_2 source saturated with water at the vapor pressure of the dialysate is connected to the 25 gauge needle.

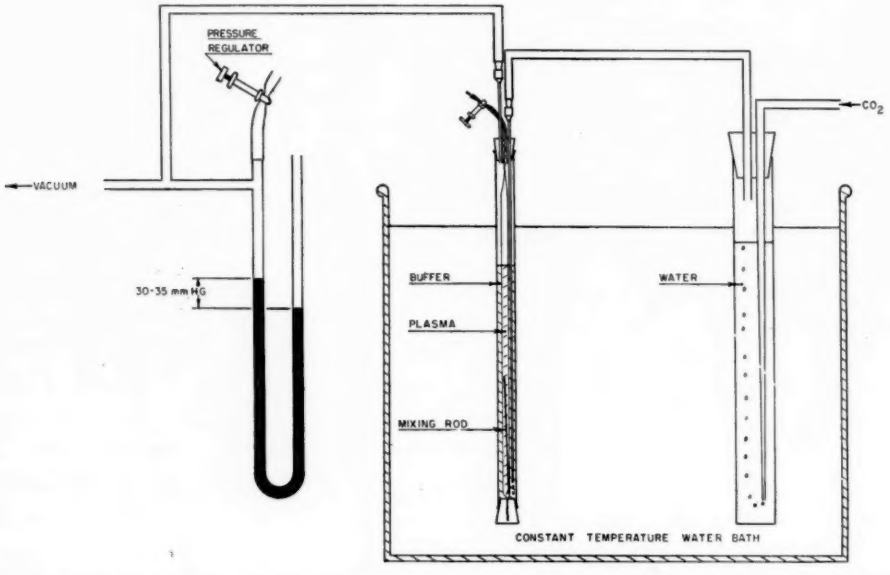

Fig. 2.—Connections of a single dialysis unit, showing means of equilibration with CO_2-O_2 mixture and of application of negative pressure.

Fig. 3 shows the essential features of a rocker-mixing assembly which holds 10 dialysis units in a Warburg water bath. The rocker is powered by compressed air acting through a truck-type windshield-wiper unit. Total excursion is roughly 60 degrees. A mixing rod inside each dialysis bag, gas bubbling through the surrounding dialysate, and continuous rocking adequately mix the solutions and continually renew the layers in contact with the dialyzing membrane. Temperature also is well controlled. The CO_2-O_2 gas mixture is so chosen with respect to the bicarbonate concentrations of dialysate and plasma that it will maintain the pH within the desired range. The ability to vary both pCO_2 and bicarbonate concentration enables one to determine the effect of either a respiratory or metabolic type of acidosis or alkalosis on diffusibility of plasma solutes. A negative pressure of 30 to 35 mm. Hg is applied outside the dialyzing membrane to balance the oncotic pressure of the plasma proteins and to prevent an increase of volume within the bag.

Disassembly and Collection of Samples.—When dialysis is completed, a unit is taken from the bath, the top clamp is removed, and the upper stopper is slipped free. As shown at the upper right of Fig. 1, *B*, the cellulose tubing is held by the forefinger and the dialysate is poured off. The dialysis tube and lower stopper are then removed from the glass tube and wiped dry. The dialysis tube is opened with scissors and its contents drained, as shown in the lower part of Fig. 1, *B*. The upper stopper with its polyethylene and needle inserts is saved and reused repeatedly. The lower stopper is discarded.

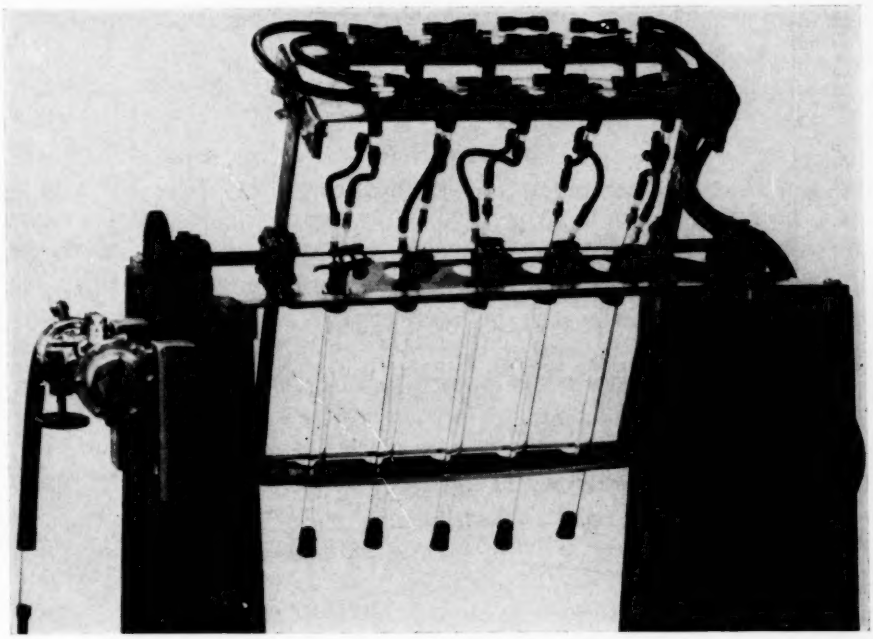

Fig. 3.—Rocker-mixing assembly which holds 10 dialysis units. Five units are shown in place. The rocker assembly is immersed in a rectangular water bath of the type formerly used for Warburg microrespirometers. The two plywood supports stand on either side of the water bath. A truck-type windshield wiper rocks the assembly through a 60° arc.

Although it is possible to dialyze with no appreciable change in volume, it is necessary for accuracy to analyze both plasma and dialysate for the solute under study. It is also wise to analyze the plasma for protein following dialysis. If the dialysis must be prolonged to attain equilibrium, one should add an antibiotic to inhibit bacterial growth. Others in our laboratory have utilized this system to study plasma binding of phenol red, radioiodine as sodium iodide, and radiomercury as Chlormerodrin, and have found it highly satisfactory. Our own immediate use has been that of studying factors affecting diffusibility of plasma calcium.

DIALYSIS OF PLASMA CALCIUM

Method.—In all of the experiments described below, the pCO_2 of the plasma-dialysate system has been stabilized at 40 mm. Hg. The concentration of bicarbonate in the fluid against which the plasma was dialyzed was varied to attain final total concentration of approximately 10 and 24 meq. per liter. The lower concentration yielded a pH of 7.0, the higher, a pH of 7.4. Calcium chloride was added to the dialysis solution in greater or lesser amounts to obtain final plasma concentrations varying between 6 and 36 per cent. Other ions were added in the proportions and concentrations roughly

equivalent to those existing in normal plasma. Calcium was determined in both the dialysate and in the plasma following dialysis by the indirect flame photometric method of Poulos and Pitts.[12]

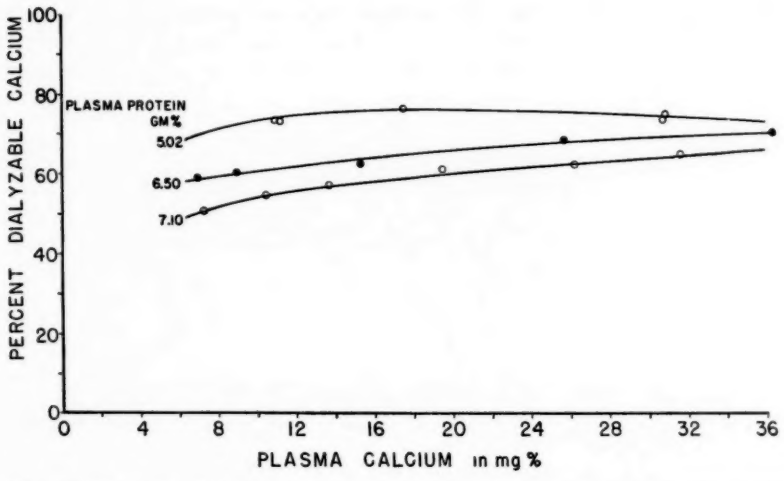

Fig. 4.—Per cent dialyzable calcium as a function of plasma calcium concentration in three samples of plasma having protein contents of 5.02, 6.50, and 7.10 Gm. per cent. All dialyses carried out at pH 7.4.

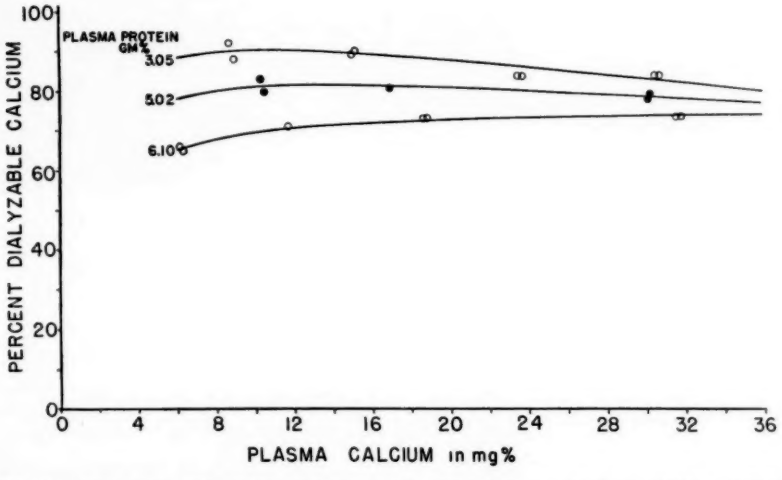

Fig. 5.—Per cent dialyzable calcium as a function of plasma calcium concentration in three samples of plasma having protein contents of 3.05, 5.02, and 6.10 Gm. per cent. All dialyses carried out at pH 7.0.

Results.—In preliminary tests it was observed that equilibrium between inside and outside aqueous solutions across the dialyzing membrane was attained in one hour. For plasma the time required was somewhat greater, perhaps twice that for aqueous solutions. Therefore we have routinely dialyzed plasma for four hours. Antibiotics were not added, since we did not feel that their need was indicated in view of observed constancy of binding at 3, 4, and 5 hours.

In Figs. 4 and 5, per cent dialyzable calcium has been expressed as a function of plasma total calcium concentration. Each point on these graphs is based on the average of duplicate analysis of both dialysate and plasma. In order that per cent dialyzable might be calculated, the observed concentration of dialysate, times 100, is divided by the observed concentration of plasma, both having been measured after 4 hours' equilibration.

Preliminary experiments indicated that no significant amount of calcium was lost by adsorption on the cellulose membrane. After dialysis, all the calcium known to be present in each unit was accounted for by analysis of plasma and dialysate, assuming that neither solution changed in volume. Within the limits of our capacity to estimate these volumes, the assumption is valid.

In Fig. 4 is presented the relation between the per cent dialyzable calcium and the total plasma calcium concentration for three samples of dog plasma having protein contents of 5.02, 6.50, and 7.10 Gm. per cent. All measurements were made at pH 7.4. The per cent dialyzable calcium increased with decreasing protein content of the plasma. The variation in binding as a function of plasma calcium concentration was greater with plasma of high protein content than with plasma of low protein content.

In Fig. 5 are presented results obtained in a similar study on three plasma samples at pH 7.0. The sample having a protein content of 5.02 Gm. per cent was the same as the one shown in Fig. 4. The lower pH was obtained by dialyzing the plasma against a balanced salt solution containing less bicarbonate. In general, calcium is more diffusible at the lower pH and the variation in binding as a function of concentration is less than at pH 7.4. It is doubtful whether the per cent dialyzable calcium varied significantly as a function of calcium concentration in the two samples of low protein content. In fact, in the plasma sample containing 3.05 Gm. per cent protein,* the per cent dialyzable calcium appeared to decrease with increasing concentration, although the change is equivocal.

The data presented in Figs. 4 and 5 have been combined and an enlarged version of the resultant graph has been used to estimate the dialyzable fraction in our studies on excretion and reabsorption of calcium in the dog.

DISCUSSION

McLean and Hastings[4] calculated the effect of pH upon the ionization of calcium in serum as $\triangle Ca^{++}/\triangle pH = -0.36$ mM calcium ion per liter per unit change in pH, but they were unable to demonstrate this difference experimentally.† We have calculated the change in dialyzable calcium per unit change in pH from data derived from studies on two plasma samples at pH 7.0 and pH 7.4 and at three different levels of calcium concentration. These data are presented in Table I. The mean value for $\triangle$ dialyzable Ca/

*The protein content of this sample of plasma was reduced by dilution with balanced salt solution.

†They assumed a protein concentration of 7.0 Gm. per cent. A/G ratio of 1.8, total calcium of 2.9 mM per kilo of water, a pH change from 7.35 to 7.60, and used the factors of Van Slyke, Hastings, Hiller, and Sendroy.[11]

TABLE I. CHANGE IN PER CENT DIALYZABLE CALCIUM WITH CHANGE IN pH OVER A RANGE FROM 7.0 TO 7.4 IN TWO SAMPLES OF PLASMA

PLASMA PROTEIN (GM. %)	pH 7.00		pH 7.40		CHANGE IN DIALYZABLE Ca (%)	△ DIAL. Ca / △ 0.1 pH (%)	△ DIAL. Ca / △ pH (mM Ca/L.)
	PLASMA Ca (MG. %)	DIALYZABLE Ca (%)	PLASMA Ca (MG. %)	DIALYZABLE Ca (%)			
5.02	10.59	79.7	11.14	73.2	−6.5	−1.6	−0.20
	10.34	83.4	10.99	73.7	−9.7	−2.6	−0.32
	16.96	80.7	17.58	76.4	−4.3	−1.1	−0.14
	30.13	77.3	30.73	74.0	−3.3	−0.8	−0.37
	30.30	78.7	30.73	73.7	−5.0	−1.2	−0.75
6.38	6.22	67.3	6.52	58.6	−8.7	−2.2	−0.22
	12.74	71.4	13.69	63.7	−7.7	−1.9	−0.24
	26.64	73.4	27.46	67.5	−5.9	−1.4	−0.64
Mean						−1.6	−0.36

△ pH = −0.36 mM free calcium per liter per unit change in pH. Although the terms "ionized and diffusible calcium" are not strictly equivalent, the amount of diffusible but un-ionized calcium is small ordinarily.[4] The agreement of relationships between diffusible calcium and pH which we observed and ionizable calcium and pH which McLean and Hastings calculated is such that it lends some credence to our measurements and their mathematical treatment. Our range of values, however, is great.

Hopkins, Howard, and Eisenberg[10] maintain that ultrafilterable calcium is independent of plasma calcium concentration below a level of 20 mg. per cent. Our findings obviously differ from these, and for this difference, we have no adequate explanation.

SUMMARY

A system of compensated dialysis is described for determining the diffusible fraction of any solute in plasma The apparatus is simple, inexpensive, and easily constructed and permits exact control of pH and temperature. Because of high surface to volume ratio, equilibrium between plasma and dialysate for most substances is rapidly attained.

A study has been made of the fraction of plasma calcium which is dialyzable and the way this fraction varies as a function of pH, total protein concentration, and plasma calcium concentration.

I wish to express my appreciation to Dr. Robert F. Pitts for assistance in performing this work and in preparing the manuscript.

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AN INDIRECT FLAME PHOTOMETRIC METHOD FOR CALCIUM IN PLASMA AND URINE

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THE determination of plasma calcium by direct flame photometry is simple and rapid. However, precision is attained only by the use of compensated standards containing sodium, potassium, magnesium, and phosphate,[1-4] in addition to calcium. Such methods cannot be applied to urine because variability in composition makes it impractical to compensate for interfering ions. As a prelude to a study of the renal excretion of calcium, we have developed an indirect flame photometric method which is precise and applicable to both plasma and urine. In brief, calcium is precipitated from a deproteinized filtrate by the addition of ammonium oxalate. The precipitate is washed once to remove traces of interfering ions, dissolved in dilute acid, and analyzed with a Beckman D.U. spectrophotometer equipped with a flame photometric attachment.

We have compared this method with a macrogravimetric procedure in which the calcium in 200 ml. samples of plasma or urine has been isolated and weighed as calcium carbonate.[5, 6] We have also compared the recovery of known amounts of calcium added to plasma and urine by this method, the gravimetric method, and the Kramer and Tisdall method.[7] The indirect flame photometric method is simpler and more rapid than the commonly used titrimetric[7] and gasometric procedures.[8] Because extensive washing of the precipitate is unnecessary, there is less likelihood both of random loss and of dissolving calcium oxalate during the wash procedures.

METHODS

Procedure for Plasma.—To 3 ml. of plasma in a Pyrex ignition tube, add slowly with mixing 6 ml. of 6 per cent trichloracetic acid. Centrifuge the mixture and pipette 3 ml. of the supernatant into a 12 ml. conical Pyrex centrifuge tube graduated to contain 10 ± 0.1 ml. The mark should extend completely around the tube. Add 2 ml. of 4 per cent ammonium oxalate, mix thoroughly by tapping the tube, and let stand for 30 minutes. Centrifuge for 5 minutes at a speed adequate to pack the precipitate and decant the supernatant.

Add 5 ml. of 2 per cent ammonia and mix, centrifuge, and decant as before. Add 5 drops of concentrated hydrochloric acid to dissolve the precipitate, dilute to 10 ml., cover the tube with Parafilm, and mix by repeated inversion. The final dilution of the plasma is 1:10.

Procedure for Urine.—Essentially the same procedure is employed for urine. However, the volume of urine and the volume and strength of the trichloracetic acid may be varied over wide limits, with the proviso that the final trichloracetic acid concentration should be

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4 per cent. A second condition is that the pH be favorable for the precipitation of calcium oxalate. Following the addition of ammonium oxalate, two drops of bromcresol green are added, followed by dropwise application of ammonia or hydrochloric acid until a green color results.

Preparation of Standard.—Dissolve 2.4970 Gm. of Iceland Spar in a minimum volume of dilute hydrochloric acid and dilute to one liter. This stock standard contains 1.0 mg. of calcium per milliliter. The working standard is diluted to 2.0 mg per cent of calcium. Distilled water for preparation of standards and for all dilution of plasma and urine should be derived from the same source and should be as nearly calcium-free as possible.

Flame Photometry.—A Beckman D.U. spectrophotometer with flame attachment has been used throughout. Although optimum settings must be determined for each instrument, the following may serve as a guide:

Oxygen pressure, 11 psi; acetylene pressure, 5.5 psi; slit width, 0.1 mm.; wave length, 422.7 mμ line of calcium; phototube, ultraviolet; sensitivity, adjusted to give a T reading of 75 with a 2.0 mg. per cent solution of calcium.

The background is measured with the same distilled water used for preparing the standard and for diluting the plasma and urine samples. It should not exceed 20 T. Accordingly, 2.0 mg. per cent calcium gives an absolute reading of not less than 55 T units. Since emission is linearly related to calcium concentration, following subtraction of background from both standard and unknown transmissions, the unknown concentration may be found from the equation, $(Ca)_{unknown} = 2(T)_{unknown}/(T)_{standard}$.

Gravimetric Procedure.—The macrogravimetric method used for comparative purposes has been fully described elsewhere.[5, 6] In brief, 200 ml. samples of plasma and urine are slowly ashed on a hot plate with use of fuming nitric acid and superoxol. The white ash is dissolved in hydrochloric acid and the calcium precipitated with ammonium oxalate with use of bromcresol green as an indicator. The precipitate is transferred to a previously ignited and weighed filter crucible and is ignited at 500° C. for at least two hours. The residue is weighed as calcium carbonate. Because of the large samples required, bovine plasma and human urine were used for those studies in which comparisons were made between the flame photometric and gravimetric methods.

RESULTS

Data on the reproducibility of calcium determinations by flame photometry are summarized in Table I. The number of determinations on any one sample of plasma or urine varied from two to twelve. In those cases where two determinations were made on any one sample, the "deviation" is actually the difference between the measurements. This means that the standard deviations, reported as ± 0.208 mg. per cent calcium for plasma and ± 0.091 mg. per cent calcium for urine, actually have a smaller value.

TABLE I. REPRODUCIBILITY OF CALCIUM DETERMINATIONS BY INDIRECT FLAME PHOTOMETRY

	PLASMA	URINE
Number of samples	256	64
Number of determinations	544	248
Range of concentration (mg.% Ca)	7–37*	0.5–17*
Range of deviations (mg.% Ca)	0–0.80	0–0.28
Mean deviation (mg.% Ca)	0.150	0.066
Standard deviation (mg.% Ca)	± 0.208	± 0.091

*High values were obtained in experiments on dogs infused with large quantities of calcium chloride or calcium gluconate.

Table II illustrates the recovery of calcium added to plasma or urine. The standard deviation of the per cent recovery of added calcium is ± 1.57 per cent for plasma and ± 1.70 per cent for urine. Both values are acceptable.

TABLE II. RECOVERY OF CALCIUM ADDED TO PLASMA AND URINE BY INDIRECT FLAME PHOTOMETRY

	PLASMA	URINE
NUMBER OF SAMPLES	3	4
Mean calcium concentration of samples (mg.%)	8.34 8.41 9.00	11.80 11.85 11.90 15.50
Amount of sample used (ml.)	3	3
Amount of 10 mg.% Ca standard added (ml.)	3	3
Number of determinations	28	28
Per cent recovery of added Ca		
Range	95.0–102.8	97.4–103.2
Mean	99.9	100.1
Standard deviation	± 1.57	± 1.70

The comparison of results by the gravimetric, flame photometric, and Kramer-Tisdall[7] methods for determining calcium is given in Table III. The analyses performed on two samples of bovine serum and three samples of human urine are enumerated. The gravimetric analysis was not acceptable unless the mean recovery of both standard calcium solution as well as standard plus an equal amount of unknown sample was within 3 per cent of the added

TABLE III. COMPARISON OF CALCIUM ANALYSES BY GRAVIMETRIC, INDIRECT FLAME PHOTOMETRIC, AND TITRIMETRIC METHODS

	GRAVIMETRIC ANALYSIS			FLAME PHOTOMETRY	KRAMER-TISDALL
	A 25 MG. CA STAND.	B 200 ML. OF SERUM OR URINE	A + B		
SAMPLE	(% RECOVERY)	(MG.% CA)	(% RECOVERY)	(MG.% CA)	(MG.% CA)
1. Bovine serum					
No. of analyses	2	2	2	5	4
Range	100.3–99.6	11.25–11.39	101.5–101.2	11.17–11.32	11.04–11.22
Mean	99.9	11.32	101.3	11.21	11.11
2. Bovine serum					
No. of analyses	2	4	3	10	10
Range	100.2–100.4	9.53–9.66	101.7–102.7	9.39–9.66	9.47–9.66
Mean	100.3	9.58	102.3	9.50	9.58
3. Human urine					
No. of analyses	2	5	4	10	5
Range	100.6–100.2	16.40–16.64	100.0–101.8	16.22–16.44	16.98–17.55
Mean	100.4	16.49	101.2	16.33	17.21
4. Human urine					
No. of analyses	2	6	4	10	
Range	101.0–100.1	16.45–16.71	99.7–102.8	16.30–16.44	
Mean	100.5	16.57	101.1	16.37	
5. Human urine					
No. of analyses	2	6	4	10	
Range	99.8–99.3	17.35–17.86	100.5–104.0	17.14–17.40	
Mean	99.5	17.64	102.7	17.27	

calcium. A comparison of the mean concentration of calcium obtained by each method indicates remarkable agreement. A more complete statistical analysis was not done because the errors due to the coprecipitation of oxalates other than calcium and the amount of calcium in the reagents were not determined.[9] However, the results obtained from each method agree reasonably well without introducing more exact correction factors, and the accuracy of the flame photometry is acceptable.

SUMMARY

A flame photometric method for determining calcium in plasma and urine is described. Its precision is equal to or better than that of the commonly used titrimetric and colorimetric methods, while it has the virtue of being somewhat simple and less time consuming. In brief, calcium is precipitated as the oxalate from trichloracetic acid filtrates of plasma and urine. The precipitate is washed once to remove interfering ions, dissolved in dilute acid, and analyzed on a Beckman D.U. spectrophotometer with a flame photometric attachment.

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CONTINUOUS AUTOMATIC RECORDING OF FIBRIN FORMATION AND FIBRINOLYSIS: A VALUABLE TOOL FOR COAGULATION RESEARCH

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PRACTICALLY all clotting studies are based upon observations made at an arbitrarily chosen moment during fibrin formation. This may be the time of the appearance of the first fibrin strand or the "endpoint" of clot formation when a test tube can be inverted. These observations provide insufficient information concerning the quality of the clot and the speed and intensity with which fibrin formation, once it has started, proceeds to completion. Our knowledge of this important part of the clotting process is limited, since no clinical devices were available to measure these factors.

The so-called thrombelastograph, designed by Hartert,[1] provides more information on fibrin formation than any other method. It has been a valuable tool in our investigations on clotting. This instrument can record continuously and simultaneously fibrin formation of three blood or plasma specimens and it provides a permanent record.

This paper describes briefly the thrombelastograph and its uses. Several illustrative examples are given. The principle of the instrument is shown in Fig. 1. A cylindrical cup made of stainless steel (to avoid extrinsic retraction-producing forces), which holds 0.36 ml. of the material to be clotted, is mounted in a thermostat and is given an oscillatory rotation on its vertical axis of 4° 45′ every nine seconds. Suspended in the cup is a cylinder of stainless steel which has a clearance of approximately 1.0 mm. from the side and the bottom of the cup. This small clearance and the limited excursion of the cup reduce to a minimum the mechanical distortion of the clot. The cylinder is suspended by a fine wire which bears a small mirror. During the process of fibrin formation, the cylinder is increasingly firmly bound to the cup as fibrin formation proceeds. The rotational oscillation of the cup is therefore increasingly conveyed to the cylinder suspended in it as fibrin formation proceeds.

A beam of light projected on the mirror is deviated as soon as the cylinder starts moving. By this mechanism, fibrin formation is converted into oscillation of a beam of light. Hence, when no fibrin is formed the movement of the cup is not transmitted to the suspended cylinder and no oscillations of the beam of light are produced, represented on the resulting coagulogram as a

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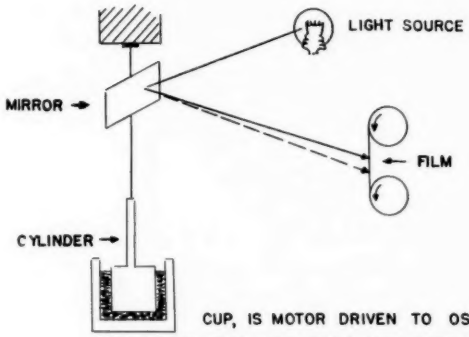

Fig. 1.—Schematized mechanism of the "thrombelastograph."

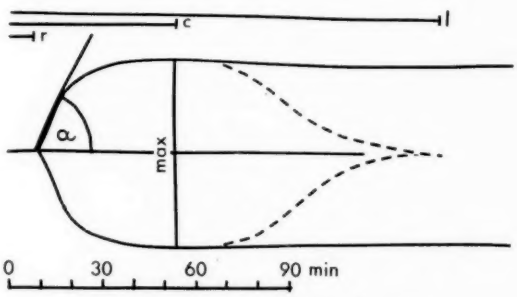

Fig. 2.—Schematized coagulogram.

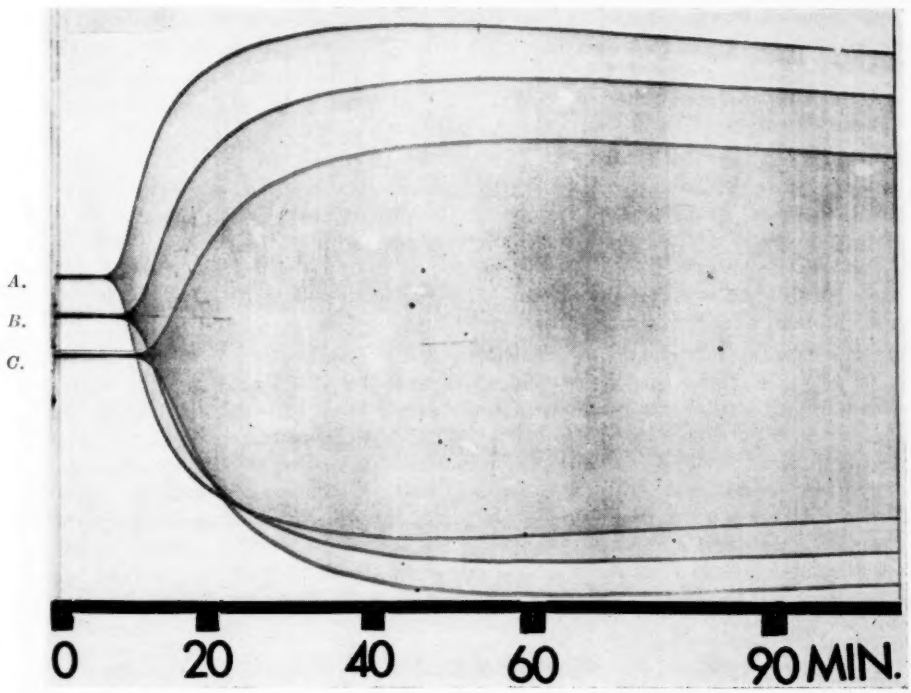

Fig. 3.—Photograph of an original film with three normal coagulograms. Time scale was added later.

straight line (Fig. 2). When fibrin begins to be formed, the beam of light begins to oscillate, thus producing a darkening of the film, the amplitude of which corresponds to the extent of the excursions of the beam of light (Fig. 3). The area traversed by the oscillating beam of light is outlined on the film by an arrangement which stops the beam at the end of each excursion for one second before swinging in the opposite direction. Thus a black line which frames the area darkened by the oscillating beam of light is produced on the film, which moves 1 mm. a minute. The time interval between the drawing of blood or its recalcification and the starting of the machine should be added. With practice, this can be reduced to a few seconds. Siliconized equipment must be used when working with blood drawn without anticoagulant. Most of our experiments have been performed with recalcified plasma, since some time lag was inevitable.

PROCEDURE

We use one part of 3.8 per cent sodium citrate U.S.P. to four parts of blood. The blood is drawn by the two-syringe technique into a syringe filled with the necessary aliquot of citrate, mixed, and spun for five minutes at 1,500 g. The cup is filled with 0.33 ml. of plasma, and 0.03 ml. 0.5 M $CaCl_2$ is added with a serologic pipette. This small volume of $CaCl_2$ reduces to a minimum the dilution effects which may mask the presence of inhibitors. The mixture is stirred with a small glass rod and the machine is started. Standardized procedures must be applied in preparing specimens for the apparatus, especially regarding centrifugation, since the shape of the resulting coagulogram* is influenced by the number and function of the thrombocytes.[3] This factor has been clearly demonstrated in experiments in which the number of platelets was reduced by differential centrifugation. This relationship to number of platelets is helpful for the coagulographic analysis of thrombocytopathias. In addition, absolutely fresh blood or plasma should be used because only then do the coagulograms have their "natural" shape. The older the specimens the smaller the amplitude in the recordings. This decrease is demonstrable within a few hours of storage and becomes pronounced after refrigeration of the plasma for 24 hours.

Previously we have found[3] that a small amount of normal fibrin must be formed before the movements of the cup are transmitted to the cylinder (Fig. 1). Extremely soft clots (those produced by trypsin or very small amounts of fibrin) are either not recorded at all or coagulograms of extremely small amplitude are obtained, since the clot does not possess the firmness necessary to bind the cylinder to the oscillating cup. The possibility of differentiating clots of different degrees of firmness is considered to be one of the advantages of this device.

Fig. 2 is a schematic coagulogram illustrating the characteristic pattern. A straight line begins to register as soon as the machine is started and indicates that no fibrin has as yet been formed and that the movements of the cup have not yet been transmitted to the cylinder. This portion of the coagulogram is called the reaction time (r). As soon as fibrin begins to form, the line begins to divide and the band becomes progressively broader as fibrin formation proceeds. The greatest amplitude of the coagulogram is defined as "max." Beyond this point no more or only traces of fibrin are formed (as shown by addition of thrombin), so that no further changes in the coagulogram occur. The value "max" reflects the firmness of the clot which is determined by the number of platelets participating in the clotting process as well as the amount of fibrin formed. This is our present interpretation. There is no good explanation for the frequently observed narrowing of

*The designer designated the clotting recordings as "thrombelastograms." We prefer the designation "coagulogram" since in our opinion the machine is not recording "the elasticity of a thrombus." For this and other reasons we have also used designations for the analysis of the coagulograms that are somewhat different from those used by Hartert, but are essentially in accordance with Della Santa.[2]

the coagulogram after "max" in specimens which do not exhibit fibrinolysis. Hartert believes that this phenomenon may be due to retraction. The time elapsed from the beginning of the coagulogram to "max" is designated the coagulation time (c): c-r is then the time required from the start of fibrin formation to its completion (as defined by "max"). The kinetics of fibrin formation is indicated by the angle α formed by the middle axis of the coagulogram and a tangent drawn from the end of r to the slope of the dividing (fibrin formation) line. The greater the value of α the more rapid is fibrin formation. The apparatus also records fibrinolysis time (l) and, in particular, permits visualization of the kinetics of fibrinolysis. The usual values for fresh, normal, citrated plasma, recalcified as indicated above, are: r = 5-10 min., c = 30-60 min., α = 57-75°, max = 50-65 mm. Alpha is somewhat smaller when whole blood is used than when the coagulogram is obtained from recalcified citrated plasma.

Fig. 3 is a reproduction of an original coagulogram showing the clotting of three normal recalcified human plasma specimens. In Graph A, fibrin formation starts after six minutes (r = 6) and is complete in 44 minutes; c = 44; α = 70°, max = 64. The values for Graph B are: r = 8, c = 54, α = 65°, max = 60; and for Graph C: r = 10, c = 58, α = 59°, and max = 56. The kinetics of fibrin formation in all three coagulograms are normal, since the slopes show no abnormality. (Compare with other coagulograms shown below, i.e., Fig. 4, *A*.)

Hypercoagulability States.—Fig. 4, *A* is a coagulogram obtained from the blood of a woman with recurrent thrombosis and embolism. No evidence of increased coagulability was

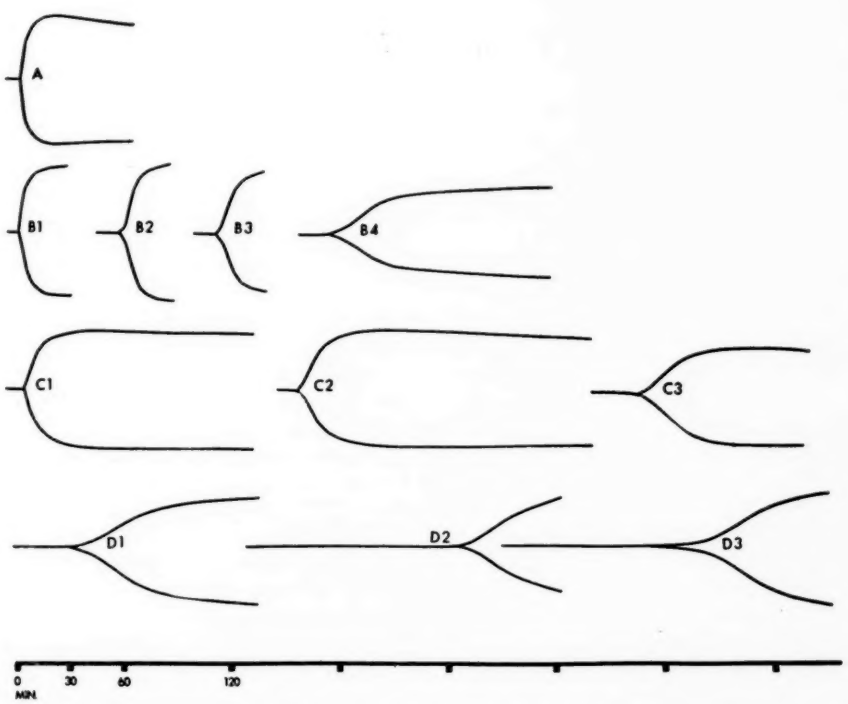

Fig. 4.—Coagulograms of hypercoagulability and induced hypoprothrombinemia. *A*, Hypercoagulability with thrombosis and embolism demonstrated by coagulogram; normal recalcification and prothrombin time.

B, 1-4, Dicumarol-induced changes of coagulograms in relation to prothrombin activity. *B 1*, prothrombin 37 per cent, *B 2*, prothrombin 27 per cent, *B 3*, prothrombin 22 per cent, *B 4*, prothrombin 10 per cent.

C, 1-3. Dicumoxan-induced changes of coagulograms in relation to prothrombin activity. *C 1*, prothrombin 30 per cent, *C 2*, prothrombin 18 per cent, *C 3*, prothrombin 7 per cent.

D. 1-3. Coagulograms of Dicumarol-induced hypoprothrombinemia. *D 2* and *D 3* were obtained from patient with bleeding tendency. *D 1*, prothrombin 7 per cent, *D 2*, prothrombin 8 per cent, *D 3*, prothrombin 7 per cent.

obtained from the usual laboratory studies, such as the recalcification time, prothrombin consumption, and prothrombin estimation (one stage diluted and undiluted plasma). It was noted, however, that Dicumarol did not reduce Factor VII below 30 per cent in spite of prothrombin levels below 20 per cent. Fig. 4, *A* was obtained between two thrombotic episodes when the patient was not on anticoagulant therapy. R is normal but fibrin formation is very fast, c being only 26 minutes, α 82°, and max 71. These values are well above normal and are seldom obtained in blood specimens from healthy individuals. Similar coagulograms have been obtained in other comparable situations. Coagulograms appear to be helpful in detecting hypercoagulability but do not reveal its nature or its origin. There is suggestive evidence that the information obtained from coagulograms bears some relation to coagulability in vivo. Eventually it may be possible to detect a thrombotic tendency by means of this method.

Regulation of Dicumarol Therapy.—The coagulogram appears to be of definite value in the control of anticoagulant therapy with Dicumarol-type drugs since it is a reflection of the over-all clotting tendency of the patient. In contrast, the prothrombin activity as determined by a one-stage prothrombin procedure does not, in all instances, reflect the actual clotting situation of the Dicumarol-treated patient, since Dicumarol-type drugs change the activity of additional factors. In the following illustrative cases, the results of the one-stage prothrombin determination have been compared with the coagulograms. Fig. 4, *B 1-4* is a serial record in a patient with acute myocardial infarction. Fig. 4, *B 1* was obtained 36 hours after infarction and 24 hours after Dicumarol was started. In spite of a reduction in the prothrombin activity to 37 per cent of normal, the coagulogram (4, *B 1*) revealed hypercoagulability ($r = 4$, $\alpha = 81°$). Further reduction of the prothrombin activity to 27 per cent produced only a relatively normal coagulogram (4, *B 2*, $r = 11$, $\alpha = 68°$). Further reduction of the prothrombin activity to 22 per cent resulted in a suggestive record of hypocoagulability (4, *B 3*, $r = 12$, $\alpha = 64°$). Only when the prothrombin time was reduced to 10 per cent did the coagulogram reveal definite evidence of hypocoagulability (4, *B 4*, $r = 16$, $\alpha = 32°$). Thus, pronounced patterns of hypocoagulability are obtained only with rather low prothrombin activities.

Similar patterns were obtained in a patient whose coagulograms are given in Fig. 4, *C 1-3*. This patient was treated with a short-acting Dicumarol derivative. Here again the prothrombin had to be reduced rather sharply to induce clear-cut changes in the coagulogram. The data are: 4, *C 1*, $r = 9$, $c = 57$, $\alpha = 72°$, max = 65, prothrombin 30 per cent), (4, *C 2*, $r = 11$, $c = 76$, $\alpha = 64°$, max = 63, prothrombin 18 per cent), (4, *C 3*, $r = 27$, $c = 85$, $\alpha = 39°$, max = 52, prothrombin 7 per cent).

Fig. 4, *D* provides some other examples. Figs. 4, *D 1* and 4, *D 2* were obtained from the same patient treated with Dicumarol, but at the time coagulogram 4, *D 2* was taken, the patient had severe conjunctival bleeding. Coagulogram 4, *D 3* is that of a patient given Dicumarol who underwent minor surgery of the skin and then bled for several days from the sutured wound prior to the taking of the coagulogram. The available data for these three coagulograms are as follows: (4, *D 1*, $r = 32$, $\alpha = 26°$, prothrombin 7 per cent), 4, *D 2*, $r = 74$, $\alpha = 21°$, prothrombin 8 per cent), (4, *D 3*, $r = 68$, $\alpha = 17°$, prothrombin 7 per cent). The coagulograms of Fig. 4 indicate that the automatic continuous recording of fibrin formation provides additional helpful information in detecting both insufficient dosage as well as overdosage in patients treated with Dicumarol drugs. In cases of very severe Dicumarol poisoning with extremely long or unmeasurable prothrombin times no coagulograms are obtainable because the clots which may be formed after hours of time are too soft, but the test tube clotting time is sometimes still measurable. Occasionally α will have values of only a few degrees and r will be extremely long.

Variations Due to the Age of the Specimens.—Fig. 5, Graph *A* was obtained from a plasma specimen with a high thrombin inhibitor titer; r is prolonged to 22 minutes. The plasma was stored for two days in a refrigerator with a resultant decrease in the amplitude; α was also reduced to 34°.

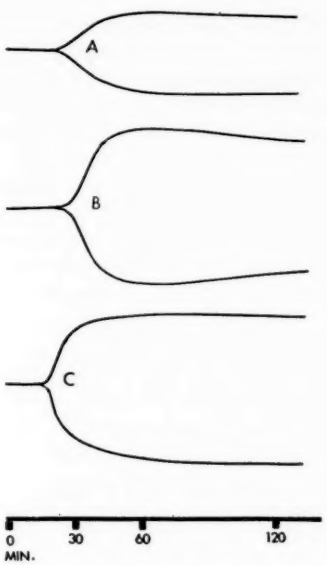

Fig. 5.—Coagulograms of plasma with different clotting abnormalities. Mutual correction. *A*, Thrombin inhibitor plasma, stored for two days. *B*, Fresh hemophilic plasma. *C*, Mixture of equal parts of A plus B.

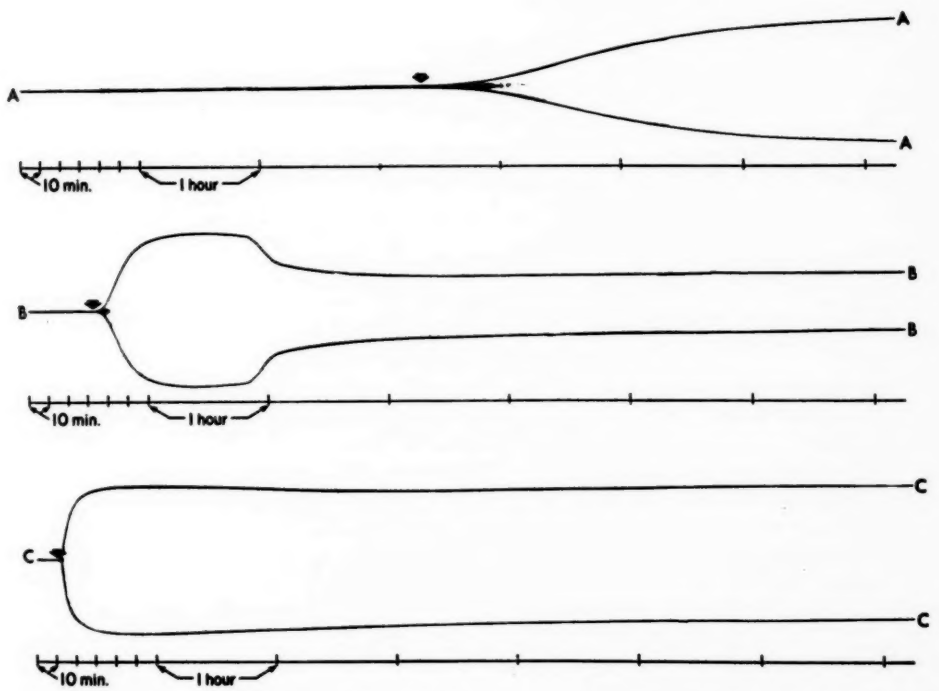

Fig. 6.—Coagulograms demonstrating the correcting effect of urine-thromboplastin on hemophilic plasma. Here an arrow is added to indicate the beginning of fibrin formation. *A*, Hemophilic control. *B*, Five micrograms urine-thromboplastin added per milliliter plasma. *C*, Twenty micrograms urine-thromboplastin added per milliliter plasma.

Studies in Hemophilia.—Fig. 5, Graph *B* is that of a case of mild hemophilia with a moderate prolongation of r to 22 minutes, α being slightly reduced to 54°. Equal parts of the plasma with the thrombin inhibitor (Graph *A*) and the hemophilic plasma were then mixed. The resultant coagulogram is shown as Graph *C*. A partial correction of the coagulation defect was obtained, r being shortened to 17 minutes, and alpha increased to 63°. Fibrin formation proceeded faster than in either the hemophilic plasma or the plasma with the inhibitor. Both plasmas had reduced values of alpha. Since these deficiencies were caused by different mechanisms, mixture of the two plasmas resulted in mutual compensation so that a kinetically normal fibrin formation resulted. The onset of this formation was, however, not entirely corrected. Fig. 6 illustrates a similar correction of abnormal onset and kinetics of fibrin formation of hemophilic plasma by the addition of a few micrograms of a potent thromboplastic material isolated from normal human urine[4]; the active material is known as the "T" fraction. Graph *A* shows the clotting pattern of a severe case of hemophilia A (r = 200 minutes, α = 12°). Addition of "T" fraction, 5 μg per milliliter (Graph *B*), resulted in a still prolonged r (34 minutes) but alpha became normal (60°), and the subsequent course of fibrin formation is almost normal. The narrowing of the coagulogram after one hour was due to mechanical artifacts caused by shaking. Addition of 20 μg per milliliter of "T" fraction (Graph 6, *C*) reduced r to 13 minutes, increased α to 82°, and restored the coagulation to a normal clotting pattern.

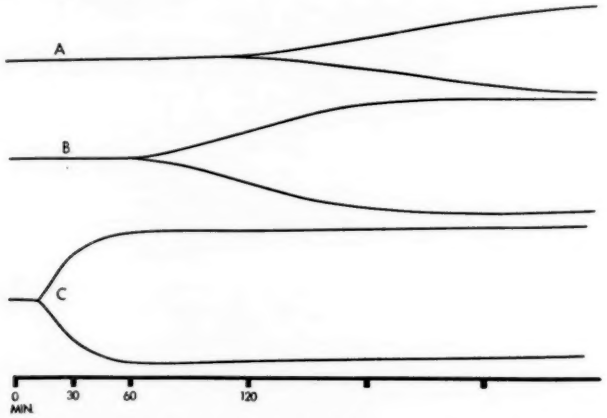

Fig. 7.—Coagulograms of plasma with circulating anticoagulant in lupus erythematosus. *A*, Untreated. *B*, 10 per cent normal fresh human plasma added. *C*, 10 per cent normal fresh rabbit plasma added.

Circulating Anticoagulant.—The coagulograms in Fig. 7 demonstrate again that the correction of a clotting abnormality does not concern only the onset of fibrin formation but also its kinetics. Blood obtained from a woman with disseminated lupus erythematosus had a very active circulating anticoagulant. Five per cent of her plasma, when added to normal plasma, prolonged its recalcification time 8 to 10 times. The thrombin time was 4 times longer than normal. Antihemophilic globulin, protamine sulfate, and toluidine blue did not have any appreciable effect in vitro on this plasma. The prothrombin time was prolonged, but Factor V activity and prothrombin consumption were normal. The clot dissolved after incubation for 24 hours. The inhibiting factor was heat resistant, not extractable by ether, and not associated with the euglobulin fraction. The patient's Wassermann reaction was positive and serum gamma globulin concentration was increased. She had severe bleeding from the nose and vagina and numerous ecchymoses. After 17 transfusions, each of 500 ml. of washed packed erythrocytes, in 21 days and daily administration of cortisone and Adrenosem, the bleeding stopped completely and the coagulograms became normal. The recalcification time gradually became shorter, and the thrombin time decreased but did not entirely return to normal. The prothrombin time became normal.

Fig. 7 shows three coagulograms from a plasma specimen obtained when the disease was at its height. Graph 6, *A* reveals extremely abnormal clotting (r = 113, c = 309, α = 7°, max = 41). The reduced value of *A* indicates very slow fibrin formation. Graph 6, *B* was obtained after 10 per cent by volume of fresh human plasma had been added. The corrective effect is minor, suggesting that transfusion might not have had any particular value (r = 70, c = 227, α = 15°, max = 52). Addition of 10 per cent fresh rabbit plasma restored clotting toward normal (r = 13, c = 66, max = 68, α = 49°, Graph 7, *C*). These results suggest that the inhibitor is specifically inhibiting human clotting components. Since rabbit and bovine components are not inhibited, these heterologous materials can be used to replace the neutralized human components and restore to normal not only clotting time but, as demonstrated by the coagulograms, the entire clotting pattern.

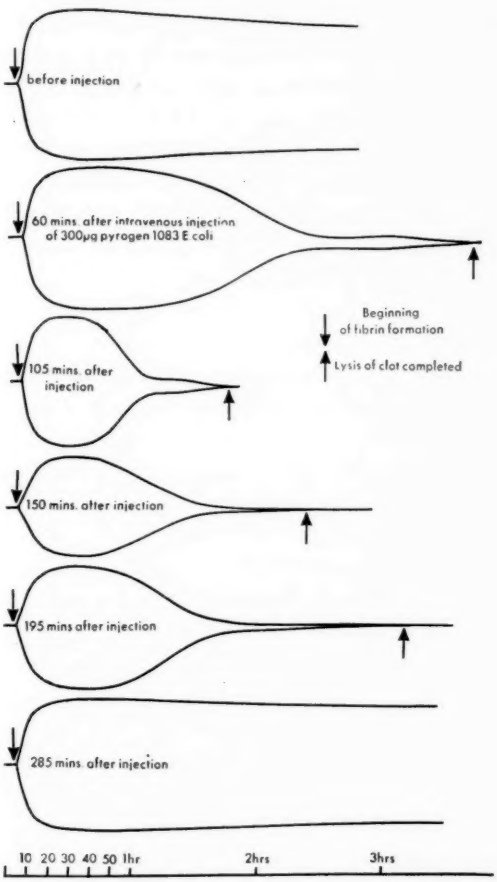
before injection
60 mins. after intravenous injection of 300μg pyrogen 1083 E coli
Beginning of fibrin formation
Lysis of clot completed
105 mins. after injection
150 mins. after injection
195 mins after injection
285 mins. after injection
10 20 30 40 50 1hr
2hrs
3hrs

Fig. 8.—Pyrogen-induced fibrinolysis: serial coagulograms.

Fibrinolysis.—Fig. 8 illustrates fibrinolysis as measured by the coagulogram. These curves were obtained with serial plasma specimens from an individual who had been given an intravenous injection of 0.3 mg. of a protein-free acetylated pyrogenic lipopolysaccharide prepared from *Escherichia coli*. These and related pyrogens induce fibrinolysis in man (but not in animals or in vitro with human plasma) by triggering the release of an activator of plasminogen.[5] The fibrinolysis is frequently associated with a reduction in antifibrinolysin activity. The coagulograms of Fig. 8 are self-explanatory. The variations in intensity of pyrogen-induced fibrinolysis at various intervals after injection are clearly demonstrated. It

was not possible to measure c and max in the coagulograms because, in most instances, fibrinolysis had started before clotting was complete. The curves show that the lysis of a fibrin clot is not a linearly progressive disintegration but that the intensity and velocity of fibrin dissolution vary considerably during the process. The general pattern is that of slow onset with gradually increasing speed, which at its maximum maintains a constant level for a variable period of time and then is followed by a gradual decline in the speed of fibrin breakdown.

DISCUSSION

The above-mentioned illustrative cases demonstrate the type of information obtainable with this apparatus which is designed for the continuous recording of fibrin formation and lysis. The device appears to be particularly well suited for investigations with blood or plasma, but less so with purified systems. The mechanical limitations preclude analysis of samples with short clotting times (less than one minute) and those which yield a weak clot. On the other hand, continuous recording of fibrin formation or disintegration is possible for hours or even days. This fact, and the ability of the device to record differences in the firmness of the clot, makes it especially suitable for studies on partial or complete fibrinolysis. A permanent record of the kinetics and intensity of fibrin formation can be obtained, and it is also possible to observe the oscillations of the beam of light during the actual recording.

SUMMARY

An instrument is described which permits the continuous recording of fibrin formation or lysis in three blood or plasma specimens simultaneously. Several illustrative cases are cited to show how the coagulogram may assist in elucidating more completely the course of fibrin formation and dissolution. Its value in research into the mechanisms of coagulation is emphasized.

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DIFFICULTIES IN ACCURATE INTRAPERITONEAL INJECTIONS IN RABBITS

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ALL modes of injections in animals are in effect forms of minor surgery, which require knowledge of anatomy and technical skill. Injections into animals are made by most workers who have learned techniques empirically, having once seen the method of using a hypodermic syringe and needle. Injections via subcutaneous and intramuscular routes are very simple, and even intravenous injections in dogs, cats, mice, and rabbits are not difficult. Intraspinal and cisternal injections on the other hand require meticulous aseptic technique, manipulative dexterity, and a sound familiarity with anatomy. Intraperitoneal injection in rabbits is by no means as simple as it seems, and it certainly is not only an act of plunging a needle into almost any abdominal area. Unless the greatest care is taken, the needle (and thus the injected mass) may not finish up in the coelomic cavity, but in one or other of the viscera or in the midst of intestinal ingesta. Manifestly, in highly critical work such as toxicity studies (or when bacteria, viruses, or parasites are injected) in which the intraperitoneal inoculations must be used, it is clear that erroneous deductions might be drawn. Through observations on anomalous results of toxicity following intraperitoneal and intravenous administrations of a compound our attention was drawn to the complexities. It was found that intraperitoneal injections of a toxic compound gave unpredictable and nonreproducible results, as opposed to the findings in the toxicity response to the same compound given intravenously.

Having observed that the needle was not properly located, we attempted to standardize the factors which might influence the needle track and its final resting place in the abdomen after intraperitoneal injections. We considered: (a) the way the rabbit was held, (b) the rigidity of the abdominal musculature, (c) the size and gauge of the needle used, (d) the depth of penetration into the abdomen, and (e) the exact anatomical site which would give the greatest ease in deposition of the injection material into the coelomic cavity proper.

Finally, it might be mentioned that the problem is obviously an old one, but there is no indication in the literature (J. Markowitz, 1949; E. H. Craigie, 1951) or by personal discussions with experimentalists that the difficulties have ever been truly appreciated. The rabbit, however, is possibly the only

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rodent used in experimental laboratories in which intraperitoneal injections can go astray, largely because of the immense bulk and length of the stomach, cecum, and large colon, which occupy most of the abdominal cavity.

METHODS AND MATERIALS

Evaluation of the fate of the intraperitoneal injections was accomplished by using 2 ml. of a concentrated solution of a water-soluble green dye for all injections, immediately following which each rabbit was sacrificed and necropsied. Successful injections into the intraperitoneal cavity were characterized by uniform distribution of the dye over the peritoneal surface of the viscera (and absorption may be followed via the lymphatics to the parietal pleura); unsuccessful injections resulted in deposition of the dye into one or another of the abdominal structures.

Fig. 1.—Showing recommended position of animal and direction of hypodermic needle for intraperitoneal injection.

Random selection of albino rabbits ranging from 1.0 to 4.5 kilograms was employed. Since the state of alimentary elimination and fasting for a 24-hour period was found to be of no consequence, these factors were subsequently ignored.

Standardization of Injection Techniques.—Eight different techniques were set up as standard testing methods to cover our experimental approach.

1. An assistant restrained the rabbit with its head downward and with the abdomen directed away from the holder in order to allow the viscera to settle toward the diaphragm. A ¼ inch 23 gauge needle was inserted to the hilt into the abdomen on either side of the ventral midline approximately ½ to 1½ inches craniad to the pelvic inlet.

2. The tip of a 1 inch 18 gauge needle was filled with solder, and a new orifice of the same caliber was drilled laterally in the shaft ½ inch from the tip. This needle was inserted to the hilt perpendicular to the abdominal wall on the ventral midline, into the midabdomen of rabbits held in the same manner as Method 1.

3. The rabbit was held downward as in the preceding methods. A ¾ inch 23 gauge needle was inserted to the hilt ½ inch lateral to the ventral midline on either side in the midabdomen and directed toward the pelvis.

4. The rabbit was held in sternal recumbency on a table. A 1½ inch 19 gauge needle was inserted into the right paralumbar fossa lateral to the transverse processes of the lumbar vertebrae and directed downward toward the ventral midline to an approximate depth of ¾ inch.

5. Method 4 was repeated with use of a 1 inch 22 gauge needle instead of the 19 gauge needle.

6. The rabbit was held as if standing on its hindlegs, with the abdomen away from the holder, to enhance the viscera dropping away from the diaphragm. A 1 inch 18 gauge needle (as used in Method 2) was inserted 1 inch from the ventral midline on the right side and ½ inch craniad to the costal arch. The needle was directed caudally through the diaphragm into the abdominal cavity and inserted to the hilt.

7. The rabbit was held as in Method 5. A ¼ inch 25 gauge needle was inserted to the hilt perpendicular to the abdominal wall ½ inch lateral and ½ inch caudad to the xiphoid cartilage.

8. The rabbit was held as in Method 6. A 1½ inch 19 gauge needle was inserted through the skin and abdominal muscle to a depth of about one-half its length, ½ to ¾ inch caudad to the xiphoid cartilage with the needle tip directed caudally so as to parallel the abdominal wall (Fig. 1).

RESULTS

The results are tabulated in Table I and may evoke surprise in many who regard intraperitoneal injections as a simple manipulation. We need only point out certain salient facts. For example, in the utilizing of Method 1, which is one commonly used, the injection was not into the peritoneal cavity in 51 of the 75 rabbits tested. It is also important to note that 31 or 61 per cent of these faulty injections resulted in deposition of the dye into the cecum. Consequently, studies based on this particular intraperitoneal technique—no matter what the compound—would clearly be of little use. The animal essentially would be receiving a drug via the alimentary canal, the content of which by its reaction or bulk alone would delay, or completely inhibit, absorption (or infectivity) of any test material.

The other results speak for themselves. In brief, Method 8 was found to give by far the best and most consistent results, and as it can be accomplished with ease it should be made generally known to experimentalists who use large numbers of rabbits. Further, it should be emphasized, that even with this method, and with great attention being paid to all aspects of good technique, failures will occur. Hence, an occasional paradoxical result from intraperitoneal injections should be fully realized.

SUMMARY

1. Intraperitoneal injections in rabbits is not a simple matter, and a needle inserted by haphazard methods may track into visceral structures (especially the cecum) and not into the peritoneal cavity proper.

TABLE I. RESULTS OF INTRAPERITONEAL INJECTIONS

METHOD	FAILURE: SITE OF DEPOSITION OF DYE								TOTAL FAILURES	TOTAL SUCCESSES	TOTAL TESTED	% SUCCESS
	SUB-CUTANEOUS TISSUE	STOMACH	PERIRENAL FAT	INTRA-MUSCULAR	THORAX	BLADDER	LIVER	CECUM				
1	7		3	6		4		31	51	24	75	32.0
2	2							17	19	21	40	52.5
3	1							12	13	12	25	48.0
4			3					8	11	14	25	56.0
5			4					9	13	12	25	48.0
6	2		8		1			4	15	25	40	62.5
7		5		3			4	2	14	18	32	57.3
8	3	7	1	1				3	15	85	100	85.0
Totals	15	12	19	10	1	4	4	86	151	211	362	

2. In critical work on the toxicity of any compound (and in work with infectious agents) in which this route is used, the possibility of erroneous results and deduction must be obvious.

3. After repeated tests done on 362 rabbits, a method was standardized in which the failure to find the cavity with the needle was very small. Failure may still occur in spite of great manipulative care and should be appreciated by all experimentalists who use intraperitoneal injections in rabbits.

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THE SEDIMENTATION OF ERYTHROCYTES AT AN ANGLE OF 45 DEGREES

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IN THE course of our studies of the growth of individual children, we have been following the changes in the blood cells for over 20 years. We became interested in the variations in the erythrocyte sedimentation rate (ESR) 18 years ago. Since then, periodically, we have taken time out of our primary investigations of growth in order to satisfy our curiosity about some of the mechanisms involved in sedimentation. Early observations of the phenomenon, as examined in capillary tubes viewed through a microscope, led us to investigate the rate of sedimentation under many different conditions. The purpose of this paper is to report on those aspects of our studies which have led us to prefer a 45° angle rather than a vertical position for the tube, in the determination of the ESR.

Several factors have made us hesitant to add to the already voluminous literature on red cell sedimentation. If one reads with care the account of the remarkably accurate observations of Sir William Hewson,[1] presented to the Royal Society in 1771, together with the published results of the extensive investigations of Fahreus,[2] starting with his first in 1918, one will realize how relatively little has been added to our fundamental understanding in the hundreds of articles since 1930. Then, also, there are too many methods being used already so that there is some confusion as to what is a "normal sedimentation rate." Finally, the ESR by itself appears to be less meaningful as we learn more about the factors which influence rouleau formation, which, in turn, determines the rate of sedimentation.[3]

In spite of these considerations, we feel that a brief report may be useful for the following reasons. Sedimentation rates will probably continue to be performed for some time by both clinicians and laboratory technicians. By any of the currently accepted techniques the ESR cannot be determined safely in less than one hour. If one wishes the most reliable figure for the rate one must ordinarily observe the tube for the better part of an hour, or occasionally even longer, to obtain the rate during the period of most rapid descent of the top margin of the red cell column. In contrast, sedimenting at a 45° angle enables one to obtain this figure for the most rapid rate in as little as 15 minutes in many bloods and almost invariably in the first 30 to 35 minutes.

In the usual methods, with the tube held in vertical position, variations in the height of the column of blood result in significant differences in the

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ESR. Such differences persist even when one records the rate as the per cent of the total column represented by the number of millimeters fall in a given time period. At a 45° angle the millimeter fall observed during the 5 minutes of most rapid sedimenting represents essentially the same per cent of the total column, no matter what the height, for columns ranging from 50 to 150 mm. The advantage of this will be obvious to anyone who has filled sedimentation tubes. A further advantage is that minor deviations of ±5° from 45° produce no significant distortion in the resulting sedimentation curve, whereas such deviations from the vertical will not infrequently produce a speeding up of the ESR. And, finally, in repeated tests on the same blood, including concentrations or dilutions of that blood, we have found greater consistency in the results when the tubes are positioned at a 45° angle than when they are vertical. Since it was our early observation of the process in capillary tubes which led us to hypothesize that the above-mentioned advantages might be found, it seems wise to start with a brief description of what we observed.

Most of these 100 observations in capillary tubes were on healthy infants, children, or adults. Heparin was always used as the anticoagulant. In about one-third of the experiments, observations were made both on whole blood and on the same blood diluted with its own plasma to cell volumes ranging from 20 to 40 per cent. Although capillary tubes do not yield a reliable ESR, yet they are ideal for observing the details of the process of sedimentation from the start of the formation of rouleaux to the final piling up of the red cells at the bottom of the tube. Considering what is known about the influence of the proteins[3]—notably fibrinogen, gamma globulin, and alpha 2 globulin—it is not surprising that one can observe differences in speed of formation and in tightness or integrity of rouleaux. We observed such differences repeatedly in different bloods or in the same blood in varying dilutions. In spite of this, the general nature of the process appeared to be similar in all bloods. Hence the account which follows will present a more or less composite picture of sedimentation as we observed it in many different bloods.

The microscope was set up and tilted at right angles to a level table. The holder for the capillary tube was a glass slide covered with black gummed tape except for a vertically running slit just wide enough to hold the tube. This was clamped to the mechanical stage so that any part of the column of blood could be observed promptly after filling. With a capillary tube of thin clear glass, with uniform lumen of approximately 0.1 mm., it was possible to visualize clearly the behavior of individual cells under high power magnification or the form and alterations in the column as a whole under lower magnification. In all instances it was possible to start observations in less than a minute after the filling of the capillary tube from a small vial of blood which had been well mixed for four minutes.

In rapidly sedimenting bloods the formation of rouleaux started almost immediately, so that by the end of the first minute there were already many small simple rouleaux, each consisting of 3 to 20 erythrocytes. Usually, the slower the start of sedimenting as viewed in a Wintrobe tube, the slower was this first formation of rouleaux seen in the capillary tube. In bloods whose sedimentation rate was average or faster there was always active formation of rouleaux by the end of the first 3 to 5 minutes. At first these increased in size by the active addition of one cell at a time, but soon small rouleaux began to combine on contact. Parallel observations of the same blood under a

cover-slip, with oil immersion lens, showed that single cells were "pulled" up onto the end of a rouleau as though by some force of attraction whose strength varied in different bloods. Small rouleaux appeared to "stick" almost anywhere along a larger one but usually with the surface of the red cell at one end of the smaller rouleau held flat against some part of the larger one.

After a lapse of 5 to 15 minutes there appeared large ungainly masses of cells which looked like disorganized clumps under low power but which, under higher magnification, were revealed as irregularly joined and many-shaped rouleaux. By the time the maximum speed of descent was recorded in a Wintrobe tube, we observed in the capillary tube mostly large clumps of erythrocytes, each consisting of a conglomerate mass of rouleaux joined in every sort of pattern. Under low-power magnification these often resembled masses of tiny worms, squeezed or moulded into innumerable shapes, never twice the same, as they twisted and turned, colliding with each other or being buffeted by the strong upward flow of plasma.

This upward flow of plasma was the most arresting part of the observation as we viewed the phenomenon for the first time. This was easy to visualize because the fluid streaming upward carried with it many single cells as well as some small rouleaux. The presence of these cells helped to reveal the impact of the upward-flowing plasma on the descending masses of rouleaux. In rapidly sedimenting bloods this flow of plasma was strong enough to carry cells several millimeters above the upper level of the settling red cell column. This is the phenomenon which results in a hazy or irregular upper margin in a very rapidly sedimenting blood in a Wintrobe or Smith tube.

In a satisfactory preparation, with a cell volume of not over 30 per cent, we found it easy to time the speed of sedimenting of a given mass of rouleaux. A large tightly packed mass which remained intact through ten minutes of descent would always sediment more rapidly than a similar small one in the same blood. For example, in one diluted blood such a mass, estimated to contain about 300 to 400 cells, averaged 32 seconds per millimeter descent, while one of approximately 50 to 60 cells averaged 75 seconds per millimeter. In this particular blood, observed many times, such masses rarely split apart. In fact, they often continued to increase in size either by catching up with smaller rouleaux, which, on contact, seemed to hold on, or by bumping into and "sticking" fast to very small rouleaux flowing upward in the current of plasma. In some other bloods, even though similarly large masses of rouleaux were formed, they showed repeated fragmentation upon colliding with other masses. In a few such bloods the rouleaux were apparently so loosely bound together that long streamers of cells would simply drop off the main mass as it floated downward through the upward flowing plasma. It is obvious that the multiple factors involved make generalizations difficult. Nevertheless one can say that usually, in bloods of approximately the same cell volume, those with the fastest ESR in a Wintrobe tube are the ones which reveal the promptest formation of tightly formed masses of rouleaux, resistant to fragmentation.

In addition one must remember that the relative amounts of plasma and cell mass play an important part in the ESR. There are two separate aspects to the influence of this relationship on the sedimentation. First, it appears that it is the amount of a given influential protein per cell which governs the rate of sedimenting.[3] Hence diluting a blood with its own plasma, thus increasing the amount of protein per cell, should increase the ESR. As expected, we observed not only an increase in the sedimentation rate but also increased speed of rouleaux formation with tighter, more compact masses of rouleaux when blood was so diluted. The second factor one might call more simply mechanical. For example, when the cell volume is over 50 per cent it is difficult for the tremendous number of cells in large rouleaux to settle while at the same time allowing an adequate channel for the displaced plasma to flow up from the lower part of the tube. In our experience the smooth descent of large masses of rouleaux was interrupted most frequently in tubes of small bore and, occasionally, even in a tube of 2.5 to 3.0 mm. lumen. By contrast, in a blood whose cell volume was reduced to 20 per cent or less, it was difficult or impossible to read the ESR. When we observed such bloods in capillary tubes, there was seen an even but sparse distribution of small to medium-sized rouleaux falling steadily through plasma which showed very little evidence of upward flow. Grossly there was no clear-cut red cell column, nor could an upper margin be defined with any accuracy.

In capillary tubes held at an exact vertical position, under low-power magnification, the red cells appeared to form a central core with a rapid flow of plasma clearly visible as a thin layer around the periphery of the erythrocyte column. With higher magnification we observed that there was also a very active upward flowing of plasma throughout the central core of massed rouleaux. The picture thus revealed was one of chaotic activity with rouleaux of all sizes and shapes following erratic courses downward, bumping into each other or being diverted by the plasma flowing upward. When the capillary tube was positioned at a 45° angle the picture revealed in the same blood was much more orderly. During the first 3 to 5 minutes, while rouleaux were being formed, there appeared an area of clear plasma along the uppermost edge of the whole length of the tube. Here we observed a smooth flow of plasma carrying upward a few single cells and an occasional tiny rouleau. Soon afterward the masses of rouleaux were observed floating downward in a more nearly straight pathway paralleling the sides of the tubes. Although there was still some interference from currents of plasma, flowing up from the dependent edge of the angled tube, yet the descent of the rouleaux was definitely faster and less wandering than that observed in the vertieally positioned tubes. The consistency of such observations on the same blood over a period of years together with the characteristic variations observed in different bloods led us to start routine sedimenting in angled Smith and Wintrobe tubes.

There have been many references during the past 30 years to alterations in the sedimentation rate resulting from any inclination of the tube away from the vertical. The earliest comments were concerned with the need to keep one's sedimentation tubes at a

constant vertical position because it had been noted over a century ago that even relatively small slanting of the tube caused an increased rate of sedimentation. In recent years a number of investigators have studied the effect of the angle of inclination on the ESR. One of the most complete discussions is that of Lundgren,[4] published in 1927. We have been unable to confirm either his observations that the "aggregates are rounded" or his concept of regular layers of rouleaux settling vertically in an angled tube. Thus his ingenious concept which led him to multiply the rate in the angled tube by the cosine of the angle to obtain the rate in the vertical tube is not logical in view of our own observations. Moreover, it failed to yield the proper correction for the majority of the bloods in our series of experiments in which tubes were held at angles varying from 15° to 90° from the horizontal.

From our observations of the sedimentation process in capillary tubes, combined with the determining of approximately 1,500 sedimentation rates carried out simultaneously in both angled and vertically held Smith or Wintrobe tubes, we feel justified in suggesting that the ESR may be determined with greater ease, speed, accuracy, and reliability when the sedimentation tube is held at a 45° angle. We realize that this raises difficult questions for anyone who would consider changing his present method. The following questions seemed important to answer: (1) Is 45° the best angle to use? (2) Is the size of the lumen of the tube of equal importance in both angled and vertical tubes? (3) Does the height of the column of blood have similar significance in the two positions? (4) Can one convert the observed ESR at a 45° angle to a reliable figure for the equivalent rate in the routine vertical position?

In a series of 36 experiments we varied the angle of the sedimentation tubes, keeping duplicate tubes at 15°, 30°, 45°, 60°, 75°, and 90° from the horizontal. Curves were constructed from 5-minute readings of the sedimentation in each tube. The ESR was recorded as millimeters fall in the 5-minute period of most rapid sedimenting. It was found in all but one instance that the ESR was fastest in the tubes positioned at a 45° angle. At angles of 15° and 30° the results were often erratic, the curve not smooth, and the duplicates checked less closely than those of any angle from 45° to 90°. At 60° and 75° the results were as reliable and consistent as at 45° but always gave a slower rate than those at 45° when the ESR was recorded as the rate during the 5 minutes of most rapid sedimenting. Moreover, as we progressed from 45° to 60° to 75° to 90° we found a steadily increasing trend for the 5-minute period of most rapid sedimenting to move from an earlier to a later point in the curve. The curves drawn from a typical experiment are shown in Fig. 1. Here it may be seen that one could be sure of having recorded the fastest period of sedimenting in the tube at a 45° angle by the end of 30 minutes. At 60° or 70° one had to wait until 40 minutes and at 90° (vertical) one was hardly sure at 60 minutes. In the more rapidly sedimenting bloods at 45° the period of fastest sedimenting may be in the first or second 5-minute period, while even in the slowest it will occur usually by the 25- to 30- or 30- to 35-minute periods. These observations led us to select 45° as the angle of choice.

Perhaps a few comments need to be made about our choice of a 5-minute reading interval. From Fig. 1 it is obvious that frequent readings are necessary when sedimenting at a 45° angle. However, even with our routine vertical technique we have found a 5-minute reading interval most reliable for comparing different bloods. The shorter the column of blood the more frequent the readings needed. But even in Wintrobe tubes, with a 100 mm. column of blood, the millimeters fall in 5 minutes may vary from 33 per cent to 40 per cent of the number of millimeters fall in 15 minutes in different bloods. The average relationship in Smith tubes with 50 mm. of blood reveals the most rapid fall in 5 minutes to be 0.39 times the most rapid fall in 15 minutes, but this varies considerably with the speed of sedimentation.

It is not quite so easy to answer the question about the effect of varying the size of the lumen of sedimentation tubes. In general the same principles apply to tubes at an angle or at the vertical position. In tubes whose bore is less than 2.0 mm. results are uncertain by any method. In tubes of uniform bore of 2.5 to 3.0 mm., well over 90 per cent of the results will be reliable providing other technical errors are avoided. In occasional bloods with high cell volume, and in which large conglomerate masses of rouleaux are formed, there may be temporary clogging of the lumen even in tubes of 2.5 to 5.0 mm. bore. From a brief series of experiments with several such bloods we concluded that one might not be free from such a hazard until one used a tube of 6.0 to 10.0 mm. bore. Such clogging occurred a little less frequently in 2.5 mm. tubes at a 45° angle than in similar tubes held vertically. It is to be noted that this phenomenon should be distinguished from that due to small clots resulting from failure to mix the blood promptly with adequate amount of anticoagulant (in this study, heparin). It may be added that one of the values of 5-minute readings lies in gaining a better opportunity to know when temporary clogging has occurred since it results in a brief period of slowing followed by increased speed of sedimenting. This distorts the normally smooth sedimentation curve.

Our third question concerns the effect of varying the height of the blood column in Wintrobe or other tubes of 2.5 mm. bore. With the tubes kept strictly vertical we have compared the rates in Smith tubes (50 mm.) with those in Wintrobe tubes (100 mm.) and the rates in two Wintrobe tubes, one filled to 50 mm. and one to 100 mm. This is shown in Fig. 2 in which we have grasped the results of 320 such observations. The ESR is recorded as the per cent of the total column represented by the number of millimeters fall of the upper margin of the red cell column during the 5 minutes of most rapid sedimenting. The picture is somewhat complicated by the varying behavior of different bloods, in most of which the rate in the 100 mm. column varies between 0.60 and 0.75 times the rate in the 50 mm. column. For clinical purposes the factor of 0.65 has yielded the most consistently satisfactory results. This is the factor we have used in combining our data from determinations in both Smith and Wintrobe tubes for comparison of rates in upright and angled positions.

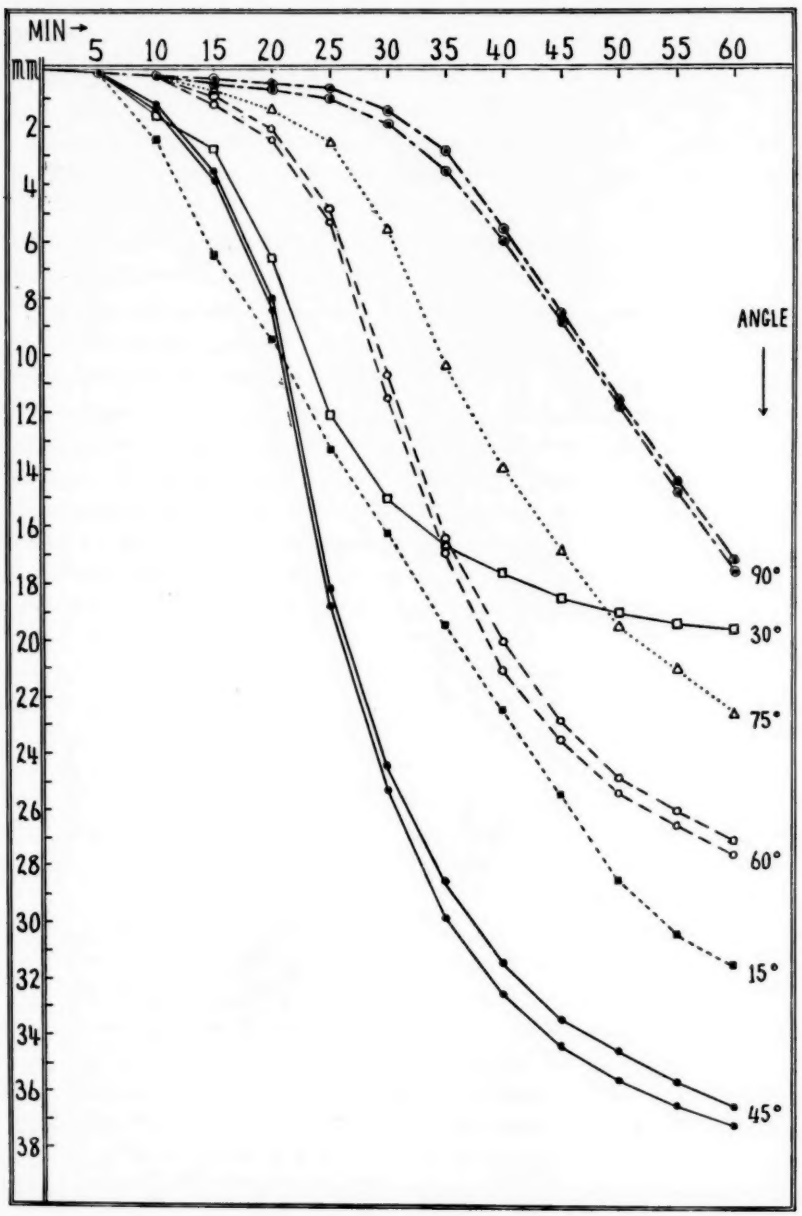

Fig. 1.—Sedimentation at different angles. Curves drawn from readings at 5-minute intervals in Wintrobe tubes containing samples of the same blood. Note that the 45° angled tubes yield a greater drop in the 5 minutes of most rapid sedimenting, and that this period occurs early in the curve.

When we examined the problem of column height as a factor in the ESR at a 45° angle, a very different picture was revealed. We found that the percentage of the column which is represented by the amount of sedimentation in the most rapid 5-minute period is essentially the same for any column

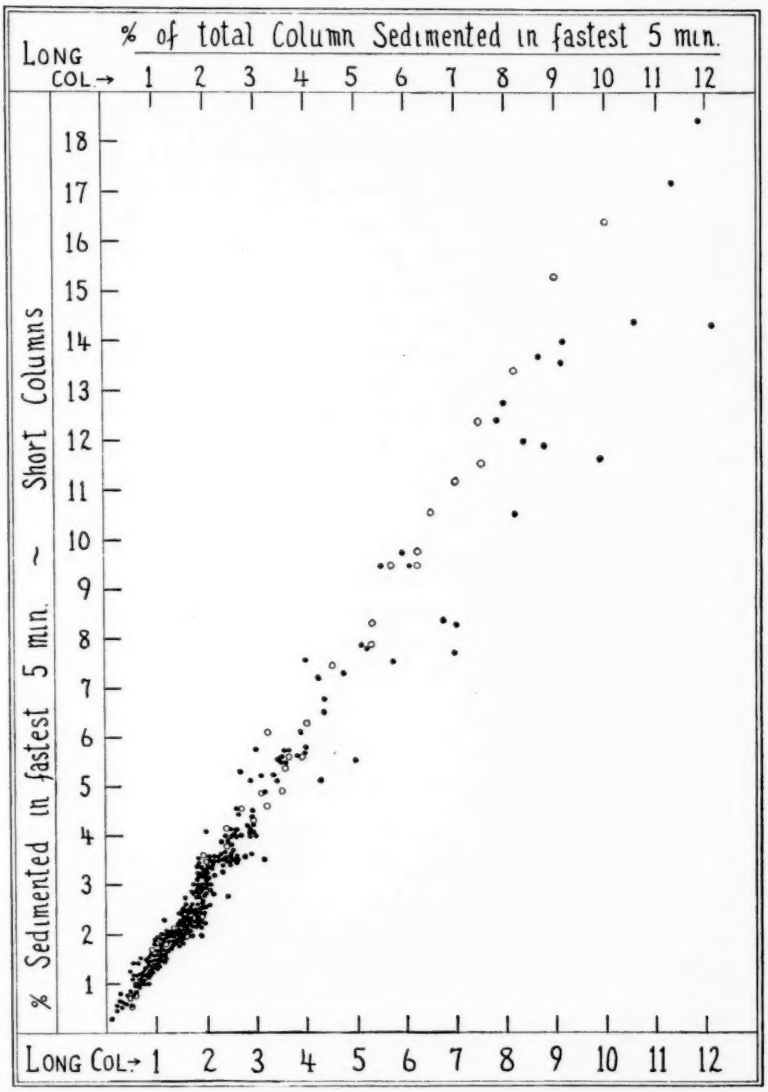
% of total Column Sedimented in fastest 5 min.
LONG COL→ 1 2 3 4 5 6 7 8 9 10 11 12
% Sedimented in fastest 5 min. ~ Short Columns
18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
LONG COL→ 1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2.—Scatter diagram showing relationship between the ESR in 50 mm. and 100 mm. columns of blood in vertically positioned tubes of the same bore. Note that the comparison is in terms of the per cent of the column falling in the 5-minute period of most rapid sedimenting.

height from 45 to 150 mm. Thus a fall of 5 mm. in the most rapid 5 minutes in a 50 mm. column turned out to be the equivalent of 10 mm. in a 100 mm. column or of 15 mm. in a 150 mm. column. A glance at Fig. 3 will reveal that some of the data shows a slight skewing, mostly in the direction of a slightly

faster rate in the shorter tubes. These were all comparisons between Smith and Wintrobe tubes. In a series of 30 observations using only Wintrobe tubes but varying the column heights from 45 to 105 mm. we found no skewing. Similarly in 10 additional experiments using tubes of uniform bore of 2.5 mm. and varying the column heights from 50 to 150 mm., we found there was no significant or consistent alteration in the percentage of the column falling in the most rapid 5 minutes of sedimenting.

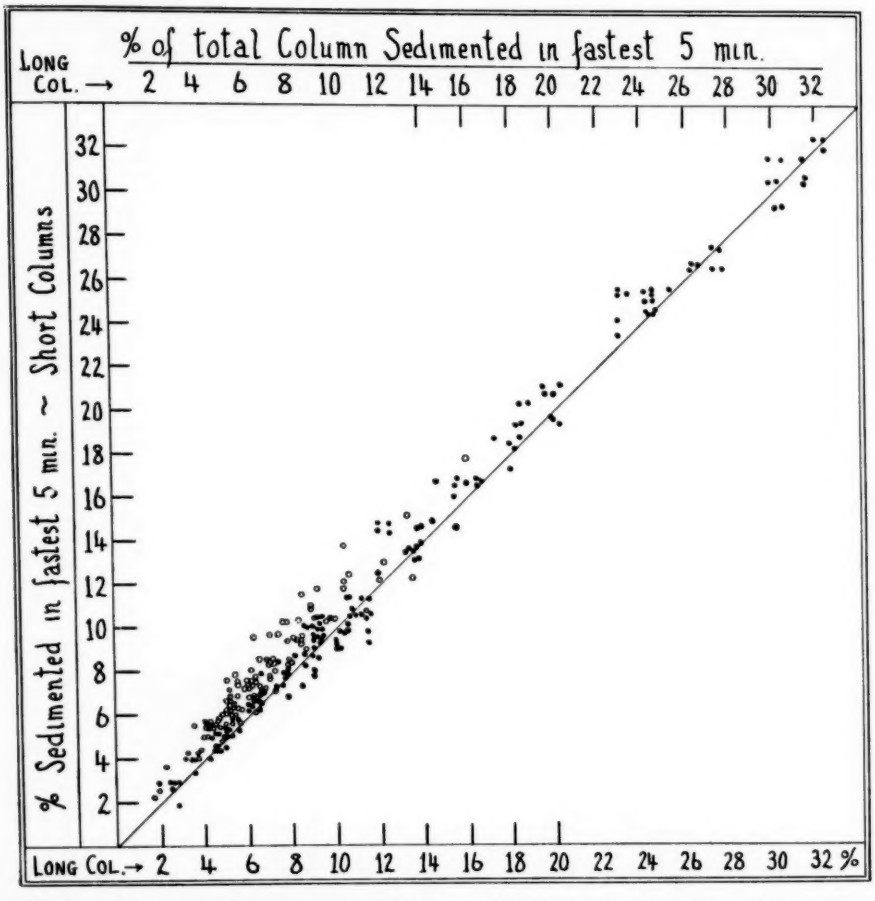

Fig. 3.—Similar to Fig. 2 except that here all tubes were kept at a 45° angle. The majority of the shorter columns of blood were approximately 50 mm. in height. Most of the longer columns were close to 100 mm., with a few ranging from 100 to 150 mm. (See text.)

The slightly faster rate in some of the Smith tubes may be dependent upon more than one factor. We have repeatedly noted a tendency for the first appearance of clear plasma at the top of the tube to occur a minute or two earlier in shorter columns. In certain bloods this might alter the fastest rate. It is also true that in the early part of our work we discovered that some of our Smith tubes were not quite constant in their bore throughout the length of the tube. Although our currently used Smith tubes are of constant bore, some of the data recorded in Fig. 3 was obtained from sedimentation rates recorded

earlier in the study. In any case the error is very small and in standard Wintrobe tubes at a 45° angle it seems safe to assume that the per cent of the column falling in the most rapid 5-minute period is the same for any column height from 50 to 105 mm. This makes angle sedimenting especially useful when only small quantities of blood are available or when it has not been possible to fill all tubes in a series to exactly the same column height.

This brings us to our final problem of how to demonstrate the relationship between the ESR in an angled tube with that in the customarily positioned vertical tube. When we originally graphed our data on 1,256 such duplicate sedimentations there was a very wide scatter. On the basis of the data presented in Fig. 2 we corrected the rates in all 50 mm. column tubes by 0.65. In addition we recorded all rates as the per cent of the total column represented by the number of millimeters descent of the upper margin of the red cell column in the most rapid 5 minutes of sedimenting. Nevertheless we were still faced with too wide a scatter for reliable prediction or conversion from the rate at an angle to the rate in the vertical tube. A study of the differences between different groups of data on infants, children, and adults by the two authors led us to re-examine the role played by the cell volume. The effect on the ESR of variations in the concentration of cells appeared to be greater in the angled tubes. On a somewhat empirical basis we corrected all the rates at an angle by multiplying each by cell volume as per cent. It is easier to obtain statistical justification* for this correction than to present physical or biologic reasons which are satisfying. Whatever the reason, when this correction was made all our data fell into quite an orderly curvilinear pattern.

Fig. 4 presents this relationship between sedimentation rates in angled and vertical tubes. All rates were recorded as the per cent of the total column represented by the number of millimeters descent of the upper margin of the red cell column in the 5-minute period of most rapid descent. The 45° angle rates are multiplied in each case by the cell volume as per cent ($\frac{CV}{100}$). The vertical rates in the 50 mm. column tubes are each multiplied by the factor of 0.65 (Fig. 2). Each circle represents the average of vertical rates for a given unit of the angle rate. The central curve drawn through these is decelerating at a uniform rate. Thus the vertical rate is 0.24 of the angle rate at 1.0, 0.36 times the angle rate at 2.0, 0.48 at 3.0, etc. The two parallel curves either side of the central curve include a zone representing ±0.5 units on the vertical scale. Eighty-six per cent of all our data falls within this zone. The number of determinations for each angle unit up to 7 is quite adequate for analysis but beyond 8 there are not enough data to validate our assumption that the relationship continues along the calculated curve drawn on Fig. 4. However, scattered data up through 12 on the angle scale suggest that our assumption is correct. Furthermore, this relationship appeared to be consistent enough for our total body of data to justify the construction of a simple nomogram for conversion of the rates from angle to vertical or vice versa.

*In this instance and in several other parts of the study of our data we are indebted to Dr. Frank McFeely, Biometrist on the Child Research Council staff, for his helpful statistical analyses.

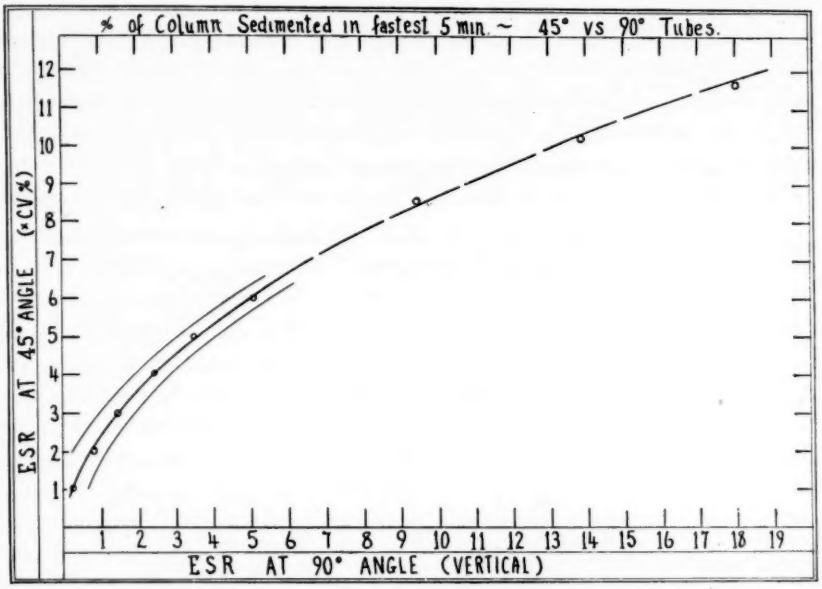

Fig. 4.—Relation between the ESR in vertically positioned tubes and in tubes at a 45° angle. As in Figs. 2 and 3 the ESR is in terms of the per cent of the total column represented by the fall in the 5-minute period of most rapid sedimenting. In addition, the rate at 45° is multiplied by C.V. as per cent and the short column vertical tube rates are multiplied by 0.65. The circles represent the means of the vertical rates at given units of the rate at 45°. The solid line is a smoothed curve through these means. (See text.)

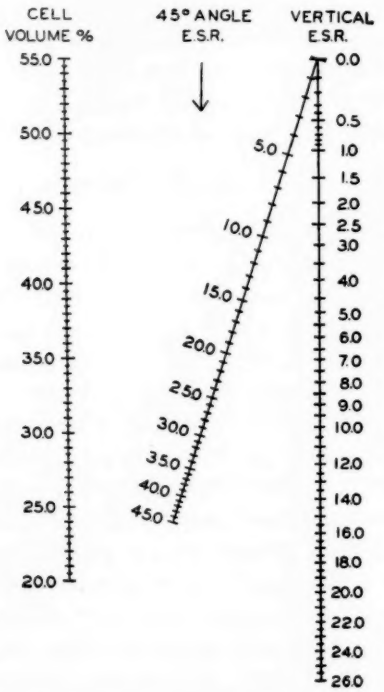

Fig. 5.—Nomogram for conversion from angle ESR to vertical (100 mm. tubes).

Such a nomogram is presented in Fig. 5. It is constructed on the basis of the curvilinear relationship shown in Fig. 4. It should be noted that on each of the two scales for the ESR, the units represent the per cent of the column falling in the 5-minute period of most rapid sedimenting. In applying it to our 1,256 determinations, we found that in 68.3 per cent of these the observed vertical rate was within ±0.3 units of that predicted by the nomogram from the angle rate and cell volume. In 85.9 per cent the predicted rate was within ±0.5 units and in 96.6 per cent of the determinations the predicted rate was within ±1.0 unit of that observed. Less than one per cent of the data yielded deviations of 2.0 units or more between observed and predicted vertical rate. Considering all the possible factors influencing erythrocyte sedimentation this would seem to be a satisfactory result. For most clinical purposes we believe this represents a safe method of converting the rate determined in a tube at a 45° angle to its equivalent for the same blood sedimenting in a Wintrobe tube in the vertical position.

As a further check on the reliability of the nomogram (Fig. 5) we have used 224 additional sedimentation rates performed since the nomogram was constructed and not used in the original analyses. These were done in duplicate Smith tubes at 45° and 90° from the horizontal. In each case the rate predicted from the angled tube was compared with the actually observed rate in the vertical. In 92 per cent the agreement between predicted and observed rates in the vertical tubes was within ±0.5 mm., while in 99 per cent it was within ±1.0 mm.

SUMMARY

We have described microscopic observations of the process of erythrocyte sedimentation as it occurred in capillary tubes. These stimulated us to investigate the possible advantages of positioning Wintrobe or Smith tubes at various angles of inclination.

The 45° angle was shown to be the most satisfactory, yielding the fastest rate for a 5-minute period. When the rate was recorded as the per cent of the total column represented by the fall in millimeters during the most rapid 5-minute period, the results were consistent and reliable.

Curves constructed from readings of the top level of the erythrocyte column every 5 minutes demonstrated also that at a 45° angle the most rapid period of sedimenting occurred earlier than at a 60°, 75°, or 90° angle from the horizontal. Except in very slowly sedimenting blood, the ESR at 45° could be measured safely by the end of the first 20 to 30 minutes.

There were 320 satisfactory observations of the same blood sedimenting in vertical position in two tubes of the same bore but one containing a 50 mm. and the other a 100 mm. column of blood. The ESR was significantly faster in the 50 mm. than in the 100 mm. columns when measured as per cent fall. A correction factor of 0.65 times the 50 mm. rate was found reasonably satisfactory for giving the equivalent rate in the 100 mm. column.

In 262 similar observations of the same blood sedimenting in tubes at an angle of 45° the ESR as per cent of total column was essentially the same no matter what the height of the column from 50 to 150 mm.

An unexpectedly uniform relationship was demonstrated when the data were graphed as the ESR at 45°, multiplied by cell volume as per cent, on the ordinate, compared with the ESR as measured in vertical position on the abscissa. The establishing of this relationship permitted the construction of a nomogram.

The results of 1,256 observations of the simultaneous sedimentation of blood in tubes at 45° and 90° (vertical) were checked on the nomogram. In 68.3 per cent of these the observed ESR at the vertical was within ±0.3 units of that predicted from the 45° angle rate and cell volume on the nomogram. In 85.9 per cent the observed was within ±0.5 units and in 96.6 per cent was within ±1.0 units of that predicted by the nomogram.

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